AD 291 692

FINAL REPORT

The Detection of Biological Aerosols by The Concentration Profile Technique

A FEASIBILITY STUDY

Period of 1 July 1961 to 31 August 1962

Contract DA 18-064-CML-2768

Submitted to

U. S. ARMY CHEMICAL CORPS BIOLOGICAL LABORATORIES
FORT DETRICK
Frederick, Maryland

MELPAR INC
A SUBSIDIARY OF WESTINGHOUSE AIR BRAKE COMPANY
3000 ARLINGTON BOULEVARD FALLS CHURCH, VIRGINIA

ASTIA AVAILABILITY NOTICES

FINAL REPORT

THE DETECTION OF BIOLOGICAL AEROSOLS

BY

THE CONCENTRATION PROFILE TECHNIQUE

A Feasibility Study

Period of 1 July 1961 to 31 August 1962

Contract DA-18-064-CML-2768

Prepared by

Hannibal de Schmertzing
Kenneth Abel

Submitted to

U.S. Army Chemical Corps Biological Laboratories
Fort Detrick
Frederick, Maryland

ABSTRACT

The purpose of this work was to test the feasibility of the concentration profile concept for the detection of airborne biological materials. The research was to include, but not be limited to: (a) the detection of chemical compounds unique to microorganisms; (b) the development of methods of analysis of such compounds sensitive to submicrogram quantities; and (c) the determination of the feasibility of performing analysis sufficiently rapid to provide information on the chemical composition of organisms to permit differentiation from other aerosol particulates.

Emphasis has been placed throughout this program on the use of gas chromatography to fulfill parts (b) and (c) of the required research. During this work, the following advances were made:

A technique was developed for the extraction of bacterial lipids with simultaneous transesterification of these lipids to methyl esters. This technique has resulted in the extraction and conversion of lipids to methyl esters in less than five minutes, compared with previous methods which required from several hours to more than two days. Conversion of the lipids to methyl esters was found to be necessary because repeated attempts to chromatograph bacterial lipids at operating temperatures up to 400°C were unsuccessful. Moreover, the technique developed for extraction-transesterification was found to be faster than any method of lipid extraction alone.

Lipid methyl ester concentration profiles were obtained for eleven microorganisms, including two pathogens. Both spore formers and vegetative bacteria were represented. The effect of nutrient, growth period, and growth form were briefly investigated. It was found that families have distinctive similarities in concentration profiles, but that recognizable differences exist among species of the same family. The effect of drastically changing the nutrient media was much less than expected. The change did result in some slight, but recognizable variations in the concentration profile. Much greater differences, both in appearance of the concentration profile and in the absolute quantity of lipid present in the bacteria, were noted when bacteria were harvested at different stages in their growth cycle. For example, bacteria harvested during the log growth phase, when cell division is proceeding at the fastest rate, were found to have more than 12 times the lipid content of bacteria in a dormant growth phase.

Some bacteria can exist in two forms: the normal, single bacterium form and a filamentous form in which dividing cell walls fail to develop during reproduction. The concentration profiles of two forms of such an organism were not found to be significantly different, although some slight differences were recognizable.

Atmospheric background was sampled, measured, and analyzed for lipid content. The background was found to be relatively constant over a three-month period, but was much higher than anticipated. The total background

varied from 2×10^{-8} g/liter to 5.3×10^{-8} g/liter. On the basis of lipid content (as estimated from gas chromatographic analysis), the biological content of the atmosphere was determined to have varied from 8×10^{-10} g/liter to 1.3×10^{-10} g/liter. This would be roughly equivalent to a variation of 1,000 to 15,000 bacteria/liter. These data do not take into account an anomalous chromatographic response which was present in all background samples. The background sample gave a peak at the position normally anticipated for a C_{22} methyl ester.

A chromatographic sampling system, capable of handling methyl ester samples of less than 10^{-10} grams without loss, was designed and initially tested. The principle of operation was varified.

Several types of gas chromatographic columns have been investigated for separating the methyl esters. These include both polar and nonpolar liquid phases. Although the polar liquid phases provide better separation and more complete differentiation, most of the work reported in this report was performed with nonpolar liquid phase, SE 30 silicone rubber. The silicone rubber was used because of the high operating temperatures required in certain experiments. These temperatures were far above the thermal stability limits of the polar liquid phases.

Three types of detectors were used in this study. The thermal conductivity detector was used as the "work horse" detector and all of the concentration profiles were obtained with it. It does not have, however, the desired sensitivity. Two commercial hydrogen-flame detectors and one commercial argon ionization detector were investigated. The Beckman hydrogen-flame detector was found to have adequate sensitivity for the determinations. With this detector, concentration profiles with as little as 10^{-10} to 10[illegible] grams of bacteria were obtainable.

Both isothermal and programmed temperature operation of the chromatographic columns were investigated. Programmed temperature chromatograms are easier to interpret than isothermal chromatograms because of the very great differences in the boiling points of the different methyl esters. It was found that the optimum temperature-programming rate is inversely proportional to the column diameter; thus, for very fast programming, Golay columns appear to offer the greatest possibilities.

Some preliminary investigations were conducted into spectrophotometric, pyrolytic, and mass spectrometric methods of analysis.

This study has verified that the concept of the concentration profile provides a feasible method for the detection and/or identification of microorganisms.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (Continued)

1. INTRODUCTION

This is the final report submitted in compliance with Contract DA 18-064-CML-2768 concerning the work performed during the period 1 July 1961 to 31 August 1962. The contractual effort is concerned with demonstrating the feasibility of using the concentration profile concept for the detection of aerosals of biological materials. This concept is based upon two premises: (a) microorganisms have a different concentration distribution of certain chemical compounds than other biological or organic materials which may form aerosol particulates and (b) required analytical methods can be developed by which the concentration distribution profile of the organisms can be distinguished. Therefore, the general purpose of the program is to develop sensitive methods of analysis for chemical compounds unique to microorganisms, and to test the feasibility of performing rapid analysis to show the chemical composition of organisms. The overall program is partly based on research by H. Wolochow.[1]

The contractual effort was divided into four tasks. Each of these tasks is discussed in the following paragraphs.

Task I consisted of planning the experimental program, literature work, and procurement of materials.

Task II consisted of the investigation of two separate approaches to the development of analytical methods for establishing concentration profiles. The first approach was based on gas chromatography. This approach has been applied to the lipid portion of bacterial matter. The second approach was based upon sensitive and unique optical absorptions or fluorescent emissions of the chemical compounds in biological material (spectrophotometric techniques).

Task III, which overlapped task II, consisted to the developmental work necessary to show the combined operation of the gas chromatographic methods developed in task II.

Task IV concerned preparation of the final report.

Gas chromatographic approaches have been found applicable to the identification and characterization of a variety of chemical and natural compounds. In gas chromatography, the rate of travel of a compound through a specially treated tube (column) is uniquely dependent upon the chemical and physical properties of the compound, as well as the properties of a selective stationary phase with which the tube is treated.

At the suggestion of Dr. Benjamin Warshowsky, Project Officer, gas chromatography was applied to the lipid portion of bacteria. Additional short studies into the feasibility of spectrophotometric methods, pyrolysis studies, and amino acid gas chromatography were also conducted.

2. BACKGROUND OF THE PROBLEM

2.1 Present Problems

The degree of infectivity associated with certain microorganisms requires their detection by feasible methods before exposure. Their virulence is associated with the fact that potentially one organism (mass of approximately 10^{-12} to 10^{-15} grams) per liter of air under optimum conditions can represent a toxic dose. This organism, being a form of life, can multiply very rapidly; a critical infective state can soon be exceeded. In practice, microorganisms would likely be found in higher concentrations (approximately 10^{-9} to 10^{-11} grams per liter). The severity of the detection problem appears to have been reduced by several orders of magnitude (compared with 10^{-12} to 10^{-15} grams per liter), but present detection capabilities of sufficient sensitivity, specificity, and rapidity for detecting the higher concentrations are still several orders of magnitude removed from the required detection methods. Therefore, adequate detection schemes still must be considered.

Various methods for the detection of airborne microorganisms are available. None, however, possesses the necessary sensitivity, specificity, and rapidity to be entirely adequate for widespread use. Bioclinical laboratory methods which are usually of sufficient sensitivity and specificity are intolerably slow because of the need for long incubation periods. Instrumental methods designed to yield more rapid results often do so at a great loss of sensitivity and specificity. However, some recent instruments have begun to satisfy the requirements; their development has progressed to the prototype stage.

Presently, two instruments exist which show potential for biological aerosol detection: the Particle Size Analyzer and the Partichrome Analyzer. It is redundant to give the characteristics of these devices at this time. They possess disadvantages, however, which seriously limit their widespread use. Expanded research efforts on new detection principles are required to establish principles for new approaches having greater capability. Such efforts must be oriented toward specific goals if the greatest advances in detection capability are to be realized at the lowest cost to the Government. A novel approach involves exploiting the bulk chemical properties of microorganisms in establishing a unique Concentration Profile for each class of organisms (or more hopefully, each pathogen). Identifications would be made by variations in the profile. This concept will be described in more detail.

The concept of the Concentration Profile is original with Melpar in its application to this detection problem. It is based on the report of a study, "Detection of Airborne Microorganisms Through Their Unique Compounds," conducted by the Naval Biological Laboratory. The study indicated the likelihood that microorganisms can be identified by qualitative detection of their unique compositional compounds.

The development of novel sensors for biological aerosols requires that the objects of the probe and the type of information the probe must sense be suitably defined. The probe must detect the presence of pathogenic microorganisms before they can infect to any extent humans in the vicinity of the probe (a dosage of one microorganism is the theoretical limit of infectivity). This means that the probe must detect minute quantities of pathogens; in a short time it must supply to a control station the information required to identify the organism type and amount. The difficulties involved in the development of such a probe are: (a) analytical techniques of sufficient sensitivity are limited in number and (b) background noise levels are high for those probes which do exist. Consequently, new schemes must be developed to detect microorganisms and signal-to-noise ratios must be increased.

There are three main stages involved in the detection of particles in an aerosol: (a) sizing, (b) concentration, and (c) assay. Sizing is necessary to eliminate spurious material such as dust, pollen, and lints. The pathogenic organisms are generally less than five microns in diameter,[2] so particles larger than this will serve only to interfere with assay techniques and must be eliminated. Particles less than five microns in diameter must then be concentrated in a much smaller volume; this volume depends on the assay method utilized. The concentrate then needs to be assayed to determine the kind and amount of pathogens.

An additional problem is the occurrence of nonpathogenic organisms in the atmosphere. Studies have shown that the number of viable organisms present in a normal outdoor environment can be expected to vary considerably.[3] In one study, it was found that the number of viable organisms varied from one organism per cubic foot to six organisms per cubic foot from hour to hour. Another longer-range study showed a variation from one organism per cubic foot to 30 organisms per cubic foot from day to day over a four-month period from February to June. The number of viable organisms is greatest during spring and early summer. These data indicate a background level of about 10^{-12} g, or approximately one viable organism per liter of air. It would be expected that nonviable organisms are present at much higher concentration levels. This could present a significant problem because chemical and/or instrumental methods would assay both viable and nonviable organisms in the particle-size range collected.

The organisms to be detected are spores (both bacteria and fungi), vegetative cells, and virus. Detection of bacteria is a lesser problem than detection of virus. Classes of bacteria have characteristic compounds which could be used in a detection system, whereas sensing the presence of virus in host cells of unknown composition is exceedingly difficult.

Assay methods will depend on the bulk chemical, surface chemical, or biological properties of the organisms. For example, the adsorption of dyes onto a bacterium involves a surface chemical property; the presence of DPA in spore formers only is a bulk chemical property and the ability of a bacteriophage to lyse *E. coli* cells is a biological property.

To be successful, a probe must include the following:

a. Sampling of large volumes of aerosol which can be concentrated in a small volume without a significant loss of particles.

b. A quantitative assay technique capable of working with 10^{-8} gram of material or less.

c. An assay which will be specific to the class of organisms of interest. For example, DPA or β-hydroxbutyric acid is unique to spore formers.

d. A process which is fully automatic.

2.2 Concept of Concentration Profile

Melpar considers that the NBL recommendation of sensitive qualitative analyses for the detection of organisms through their unique compounds can be expanded in developing a feasible detection method. This method involves the quantitative detection of those compounds common to several organisms or groups or organisms; the identification is afforded not only by what is present but also by the quantity of material present. This two-dimensional aspect releases chemical detection from the limit imposed by the requirement of unique compounds only and opens a new horizon for investigation.

Qualitative analyses for detection of organisms through their unique compounds provide a basis for rapid and specific responses. Practical application of this concept is limited, however, by the few instances in which unique constituents are associated with organisms. A broader-based concept is required if chemical analysis is to serve as the basis for the universal detection of biological materials. Melpar considers that its concept of the Concentration Profile is sufficiently extensive to provide for a practical utilization of chemical analysis.

The concept of Concentration Profile includes the use of quantitative chemical analyses of sufficient sensitivity and precision to identify several organisms which contain common constituents in varying amounts. It is unlikely that any two different pathogens would yield the same results. To provide for rapid identification, it would be necessary to select many constituents which were common to a large number of organisms and to analyze for those compounds simultaneously. Because of the anticipated variations in the concentrations of each constituent in different organisms, it is possible that specific organisms can be identified through results yielded by the several analyses. Reference profiles for pathogens, nonpathogenic organisms, and common background particulates of biological origin could provide a detection technique with a high degree of specificity which could ultimately be adapted to computer comparisons for rapid readouts.

Several apparent problems must be solved before the full potential of the Concentration Profile can be realized. These include:

a. Variations in the concentration of specific compounds in a specific agent are to be expected because of variations in the method of culture.

b. Particulate media on which agents are disbursed may distort the agent profile.

c. Mixtures of agents could possibly produce spurious profiles. These are a few of the most obvious problems.

3. RESEARCH CONDUCTED

3.1 Lipid and Fatty Acid Analysis

3.1.1 Extraction

Several solvents were used to extract the lipid components from bacteria. These were (a) methanol-ether in a 3:1 ratio, (b) chloroform, and (c) acetone.

Methanol-ether

A method described by James[4] was modified slightly. The bacteria and a 3:1 v/v mixture of methanol-ether were mixed and allowed to stand overnight. The bacteria were removed by filtration and the soluble extract evaporated to dryness in a rotary evaporator in vacuum at 40-50°. The residue was extracted three times with small volumes of petroleum ether and the insoluble material rejected. A typical extraction of *Serratia marcescens* indicated that 18 percent of the bacteria was soluble in the methanol-ether mixture, but only 4.3 percent was soluble in petroleum ether. The petroleum ether soluble material represented the lipid content of the bacteria. *Bacillus subtilis* yielded 3.2 percent lipid.

Chloroform

A chloroform-methanol mixture was used by Folch *et al.*[5] to extract lipids. The purpose of the methanol was to remove water from the material so that the chloroform could successfully extract the lipid material. Because we were working with dry bacteria, the methanol was left out and only chloroform used. Results showed that the chloroform quantitatively removed the lipid materials. The chloroform extracts contained 4.5 percent of the *Serratia marcescens* and 3.22 percent of the *Bacillus subtilis*.

Acetone

As reported by Anderson,[6] the lipids can be separated into three fractions with acetone. Anderson distinguished three lipid types: greases, phosphatides, and waxes. The greases are soluble in cold acetone, the waxes in hot (boiling) acetone, and phosphatides are insoluble in acetone. When this separation was performed on the lipids of *Serratia marcescens*, only the cold extract remained clear. The hot extract precipitated on cooling the acetone and the precipitate could not be dissolved again on heating. Because of these difficulties, the use of acetone for extraction was not considered suitable.

It was concluded that chloroform provided the most satisfactory method of extracting the lipids from dry bacteria.

3.1.2 Direct Gas Chromatography of Lipids

Preliminary attempts to analyze the lipids extracted from Serratia marcescens were made using the device shown in figure 1. The lipid extract was placed in the cup, and the unit was enclosed in the air bath oven of the gas chromatograph. The solvent was evaporated by passage of the helium carrier has through the device at room temperature. The solvent vapor was eluted through a silicone rubber/chromosorb column. After all the solvent had evaporated and been eluted from the column, the temperature was rapidly programmed to 250°C. No elution of the lipids occurred. Later, a 20 percent silicone rubber/chromosorb column 2 ft. x 1/4 in. OD was carefully conditioned to provide a column, which was stable to 400°C. Chloroform extracts from Serratia marcescens, Bacillus subtilis, Bacillus anthracis, and Pasteurella tularensis were used. Samples representing 50 mg of bacteria (about 2 mg. lipid) were injected into the flash heater of the chromatograph at 425°C. The column was temperature programmed from 125-400°C at 11°/min. No distinct elution of lipids occurred. Beginning at 375°C, however, a steady elution of material occurred. This was assumed to represent decomposition products from the lipids. Prolonged heating (several hours) of the column at 400°C resulted in removal of these materials and the re-establishment of column stability. On the basis of these experiments, it was decided that conversion of the lipids to compounds of lower molecular weight and higher vapor pressure would be required for successful chromatographic separation and identification.

3.1.3 Saponification

The saponification procedure used was that of James.[4] The diethyl ether-methanol lipid extract was refluxed with a fifty-fold excess of 2N methanolic KOH for three hours in one experiment, and 12 hours in another. Then, the methanol was evaporated, the residue taken up with water and extracted with diethyl ether after acidification.

Both the diethyl ether extract of the saponified product and the methanol, which was evaporated, were examined by gas chromatography. The amount of bacterial extract used in the experiment represented 50 mg of the original bacteria. No chromatographic response was observed. It is possible that insufficient sample was used and that chromatographic operating temperatures were too low.

To conduct this experiment more thoroughly, the saponification was repeated, starting with diethyl ether-methanol extract equivalent to 150 mg of bacteria and 6.5 mg of petroleum ether-soluble lipid. Ten ml of 2N methanolic KOH was again used, and the solution was refluxed for three hours, after which the solution was permitted to stand for 60 hours. The methanol was vacuum evaporated and retained for examination. The residue was taken up with 10 ml of water and extracted twice with 2 ml of diethyl ether, providing an alkaline ether extract. The remaining

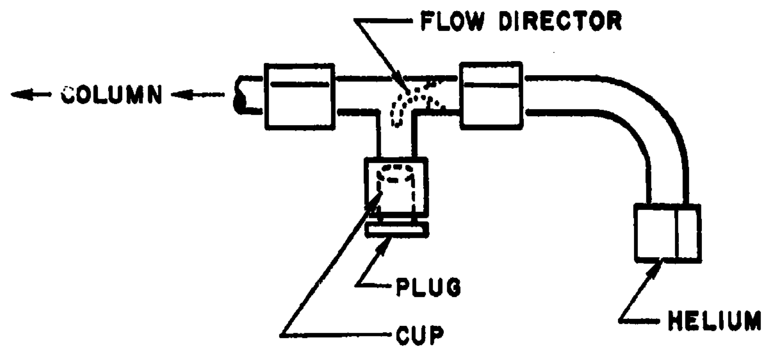

Figure 1. Solvent Evaporator

water solution, when acidified with 2N HCl, became turbid. The acidified solution was extracted twice with three ml of diethyl ether. The water solution was cleared by the ether extraction. Both the alkaline and acid ether extracts were colored yellow. The second acid-ether extract was much fainter than the first, so these were not combined. A sample evaporation and weighing of the first acid ether extract showed nearly 1 percent solids content, equivalent to about the total weight of extract used in the experiment. A sample of the first acid ether extract was evaporated on a salt disc to obtain an infrared spectra. A yellowish residue was produced. Absorption peaks appeared at 1950-2750 cm^{-1}, 1540 cm^{-1}, 1250 cm^{-1}, 1090-1010 cm^{-1}, and 790 cm^{-1}. These were generally characteristic of an acid, but they differed from the absorption in the spectrum of the original bacterial extract.

The methanol fraction and the first alkaline and acid ether extracts were examined chromatographically. The chromatographic column consisted of an 8 ft x 1/4 in. OD column packed with 20 percent by weight of silicone rubber on 60/80 mesh chromosorb W. Helium at a flow rate of 140 ml/min was used as the carrier. The injector, column, and detector were operated at 350°C. Fifty microliter samples were used, and in the case of the acid ether extract, the sample contained slightly less than 0.5 mg of solids. Neither the methanol fraction nor the alkaline ether extract produced a chromatographic response, which is the expected result if saponification is complete. The acid ether extract produced a very wide short peak extending over a retention time period of a half hour.

Subsequent experiments with mixtures of known fatty acids (palmitic and oleic) confirmed the observation above that the fatty acids do not chromatograph adequately on these columns, even at these very high temperatures.

3.1.4 Esterification of Fatty Acids

In general, esterification is a replacement reaction brought about by either an acidic or a basic catalyst. Apparently, the only exception to this is the use of diazomethane. Classical methods that use acids or bases of moderate strength are slow, requiring several hours or days for suitable yield; the replacement reaction suffers from water or other solvent competition. A long list of the various acidic catalysts in use has been compiled.[7] Several authors have studied esterification of fatty acids and transesterification of triglycerides. Kurz[8] studied direct methanolysis of triglycerides with KOH in methanol, recommending 24 hours for complete reaction, and Carter *et al.*,[9] using silver oxide with methyl iodide, recommended 36 hours. Stoffel *et al.*,[10] realizing the inhibiting effect of water, shortened the time to 2 hours, refluxing with methanol and hydrogen chloride in an anhydrous system. More recently, Hornstein *et al.*,[11] used anhydrous methanol-hydrogen chloride reagent to react rapidly with, and remove, fatty acids separated and held by ion exchange resin. The preparation of fatty acid esters from the acids in a two-minute reaction, using a reagent made by bubbling boron trifluoride into methanol, is described by Metcalf and Schmitz,[12] along with references to the origin of the reagent. A quantitative comparison of the four most used reagents for methylation of fatty acids was made by Vorbeck *et al.*[13] These reagents are: diazomethane, methanol-hydrochloric acid sublimation, methanol-hydrochloric acid with ion exchange resin, and methanol-boron trifluoride. The methanol-hydrochloric acid methods are slower, and sublimation causes loss of low-molecular-weight components. Diazomethane and boron trifluoride reagents are the most suitable for rapid and quantitative conversion. Most recently, Esposito and Swann[14] reported on the use of lithium methoxide in anhydrous methanol to transesterify carboxylic acids in alkyl and polyestercoating resins. Transesterification of these materials in two minutes was reported.

Methylation of Saponified Lipids

Most literature pertaining to procedures for methylating fatty acids starts with the acid rather than with the glyceride. The boron trifluoride technique was initially tried on the saponified lipid extract to determine its applicability.

The following procedure was used for methylation of the fatty acids: First, the lipids were saponified with methanolic KOH in the usual manner as described previously. The saponification mixture was acidified with sulfuric acid and the fatty acids extracted with petroleum ether. The ether from this extract was evaporated, and the residue dissolved in methanol. To 15 ml of the methanol solution of the fatty acids, 10 ml of boron trifluoride ethyl-ether reagent was added. This was heated in warm water for five minutes. After cooling to room temperature, the solution was poured into 100 ml of distilled water. The mixture became turbid, and it was extracted with 10 ml of ethyl-ether. The ethyl-ether layer was washed three times with 25 ml protions of distilled water. The washed ether layer was then used for gas chromatographic tests.

It was necessary to wash the ethyl-ether extract throughly with water. Otherwise, a number of gas chromatographic peaks appeared which were not caused by the lipid portion. Usually, three washes, when good boron trifluoride reagents were used, were adequate. If the reagent was impure, as many as seven or eight washes were required.

Sesame Oil

This procedure was first tried on sesame oil because of its known fatty acid composition. Figure 2 shows a typical chromatogram.

Serratia Marcescens Extract

The same treatment was applied to the chloroform extract of *Serratia marcescens* from 2.49 grams of the dry bacteria. Well-defined chromatograms were obtained.

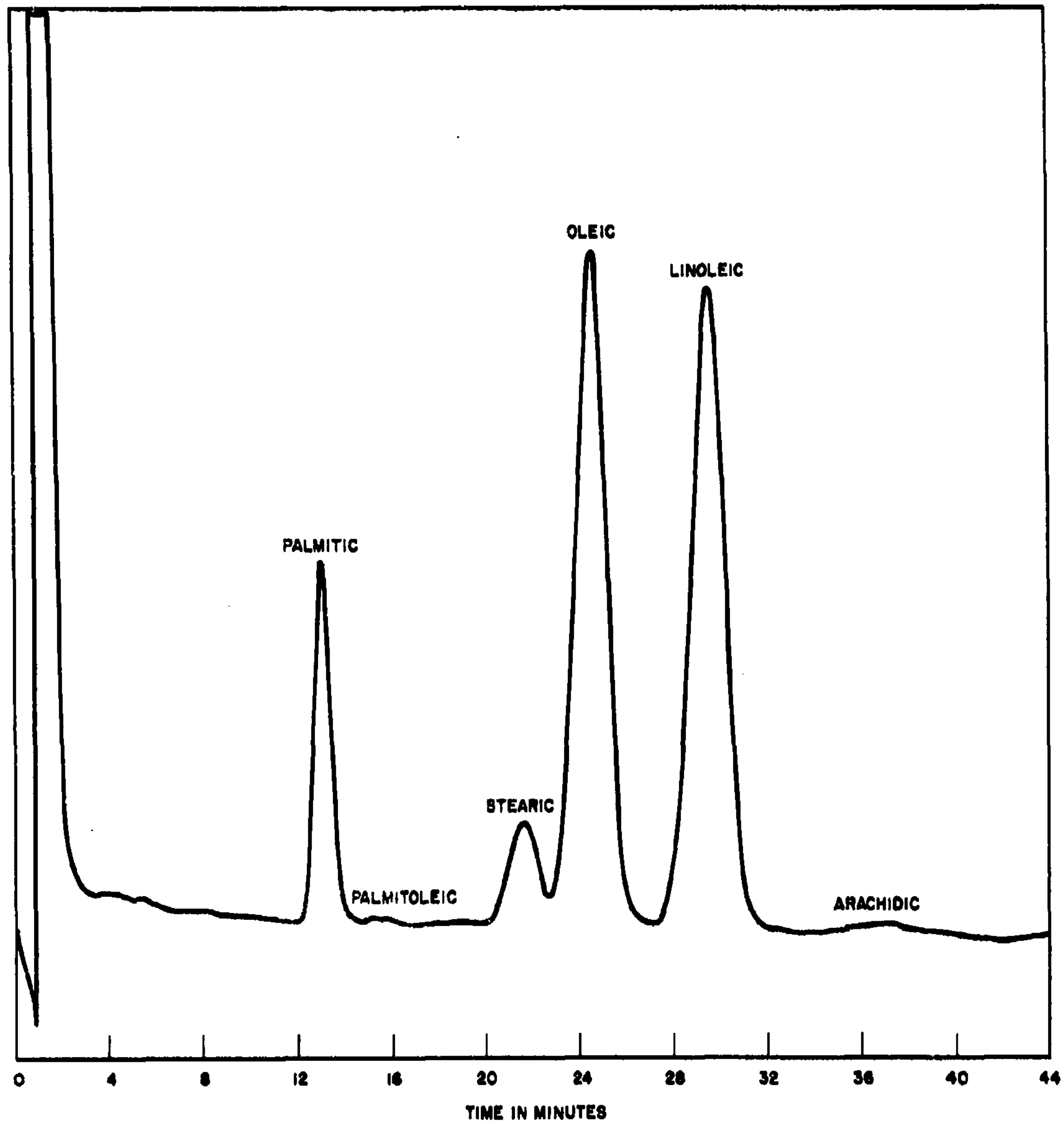

Figure 2. Methyl Esters from Sesame Oil

Column: 6 ft x 1/4'' OD containing 20% Diethylene Glycol Succinate on Type P Chromosorb, 60/80 mgh. Isothermal at 225°C. Flow rate = 100 ml/min.

Oleic Acid

A sample of technical-grade oleic acid was methylated with boron trifluoride reagent. Figure 3b shows the chromatogram obtained. It should also be noted that the material is quite impure, as indicated by the many peaks in the chromatogram.

Pimelic Acid

Pimelic acid, another fatty acid, has a low solubility in methanol and 25 ml of methanol were required to dissolve a half gram of the acid. A high degree of purity of the sample was indicated in this case.

Diaminopimelic Acid

This experiment was made to test the applicability of the method to amino acids. No esters were obtained. The diaminopimelic acid was nearly insoluble in the methanol and remained suspended, but it dissolved upon addition of the boron trifluoride reagent. This indicates that the technique as described does not work with amino acids. The subsequently successful experiment (described later) with gliadin, a protein, is in contrast to the results obtained here.

Boron Trichloride Esterification

Because of some of the difficulties encountered with boron trifluoride, such as the required extensive washing of the final ether layer, the incomplete conversion in some cases, and the refluxing required for reaction, it was thought that a more active acidic catalyst, boron trichloride, should work better. Boron trichloride is a very strong acid and reacts instantly with methanol.

Liquid Boron Trichloride

In the first experiment, it was thought that it might be possible to add liquid boron trichloride directly to the methanol. A sample of sesame oil was dissolved in a 1:1 methanol-ether mix and cooled to $0^{\circ}C$. Liquid boron trichloride at $0^{\circ}C$ was added. After three to four drops of boron trichloride were added, the generated heat ignited the solvents. After extinguishing the flames, the solution was diluted with water and extracted with ether. A chromatographic test showed that some methylation had occurred.

Gaseous Boron Trichloride

The experiment was repeated, except that, in this case, gaseous boron trichloride was bubbled directly into the methanol solution for ten minutes. In this and succeeding experiments, the procedure was the same as described for boron trifluoride, except that boron trichloride was added instead of boron trifluoride-ethyl ether, and no washing of the final ether extract was necessary.

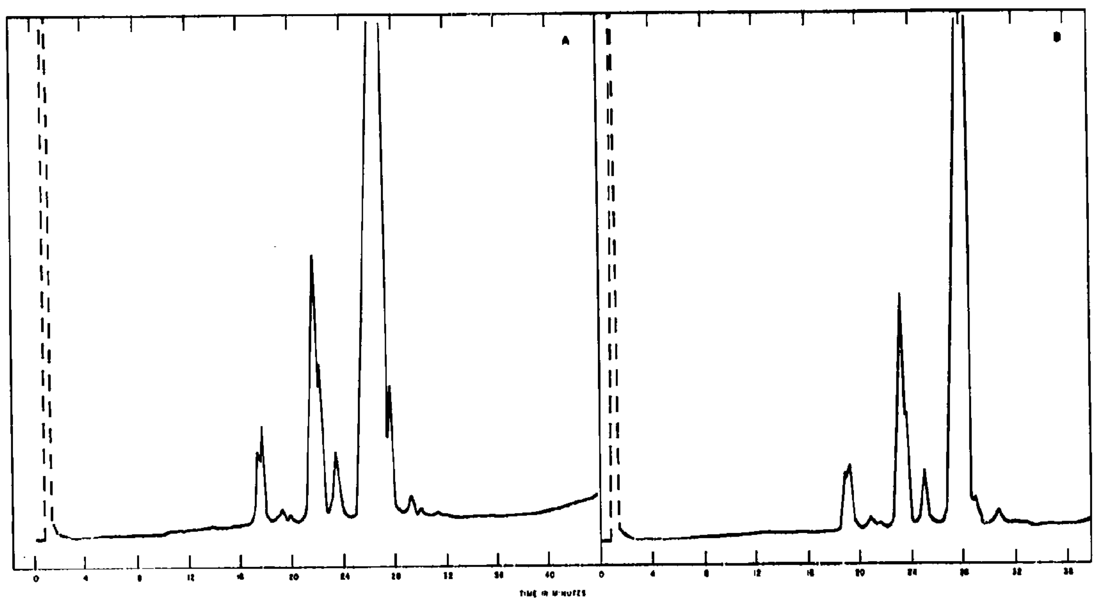

Figure 3. Esterification of Impure Oleic Acid

Conditions: 6 ft. x 1/4 in. OD column, temperature programmed 75 to 350° at 5.6°/min. 3% SR on Gas-Chrom (120/140) with free oleic acid present, helium flow = 27 ml/min. Thermal conductivity detector at 350° and 100 ma. Flash heater at 370°.

A. Direct methylation of impure oleic acid with boron trichloride in methanol.

B. Direct methylation of impure oleic acid with boron trifluoride-ether in methanol.

Sesame Oil

The experiment was carried out with sesame oil dissolved in a 1:1 mixture of methanol and ethyl ether. Nearly complete esterification of the oil was obtained. In this and the succeeding experiments with gaseous boron trichloride, the reaction proceeded smoothly without violence or overheating.

Oleic Acid

The treatment was applied to impure oleic acid in methanol. Complete conversion was obtained. (See figure 3a).

Serratia marcescens

The experiment was repeated with *Serratia marcescens* extract dissolved in methanol. Well-defined chromatograms were obtained.

Bacillus subtilis

The same experiment was conducted with *Bacillus subtilis* extracts with satisfactory results.

In these experiments with boron trichloride, the chromatographic patterns match the previous patterns obtained with boron trifluoride.

Comparison of BF_3 and BCl_3 for Methylation of Oleic Acid

The relative effectiveness of BCl_3 versus BF_3 on the methylation of this free fatty acid was also checked. No difference in effectiveness could be found.

A test to compare the reaction time for free acids was conducted as follows. A standard solution of oleic acid was prepared by dissolving 5.102 g oleic acid in 50 ml methanol. Five milliliters of this solution were taken for each test. Boron trifluoride gas was bubbled into 5 ml of methanolic solution of oleic acid for ten minutes; the extraction was processed as usual by pouring the solution into 50 ml distilled water and then extracting with ethyl ether. The same test was repeated with the BF_3 gas introduced only for two minutes, 30 seconds, and 15 seconds, respectively. These four tests were repeated again using boron trichloride instead of the boron trifluoride. All the tests (8) resulted in identical chromatograms. Both gases are very efficient catalysts for esterification of the free fatty acid; they lead to complete esterification in 15 seconds.

3.1.5 Transesterification

Two basic factors are involved in the choice of a reagent for rapid transesterification. The effective acidic strength of the catalyst is important because the stronger acids produce esters at a much more rapid rate. Also, a high concentration of the combining entity (methanol), and the absence of strongly competing substituents, particularly water, aids the reaction rate considerably, as well as shifting the equilibrium to favor ester formation. It might be assumed that boron trifluoride is the best Lewis acid catalyst of the boron trihalides because, as Pauling[15] points out, the strength of these as electron acceptors increases with decreasing atomic number of the halide. The situation is more complex, however, and Gerrard and Lappert[16] discuss another very important factor which ascribes a theoretical superiority to boron trichloride. Because of the high polarizability of the boron-chlorine bonds, boron trichloride reactions do not stop at the formation of corrdination complexes, as is the case with boron trifluoride. Instead, boron-organic compounds are formed through ionic intermediates. In the case of reaction with methanol using BCl_3, a complex forms immediately and the postulated formation of methoxy-boron bonds occurs to varying degrees with the release of hydrogen chloride. The reaction is highly exothermic. Alkyl chlorides can also be produced.

Reaction of boron trichloride with esters may involve coordination with the carbonyl oxygen followed by either acyl oxygen or alkyl oxygen fission. Sufficient information is not available to outline in detail the mechanism of transesterification with boron trichloride. In any event, the greater reactivity of boron trichloride over boron trifluoride for this purpose can be explained in terms of its behaving effectively as a stronger Lewis acid, this greater acidity arising from polarization with coordination. Boron trichloride will maintain the medium in an anhydrous state.

Comparison of BCl_3 and BF_3 on Lipid Transesterification

A comparison was made of the relative effectiveness of BF_3 gas and BCl_3 gas in the transesterification of a typical lipid, sesame oil. For this comparison, 1.266 grams of sesame oil were weighed into a 10-ml volumetric flask and made up to volume with diethyl ether. Equal portions were then transferred with a pipette into reaction flasks. Next, 4 ml of methanol were added and BCl_3 or BF_3 gas was passed into the solution for four minutes. One sample of each of the BCl_3 and BF_3 treated solutions was then allowed to remain at room temperature for 15 minutes. Each solution was then added to 50 ml of distilled water and extracted twice with 5-ml portions of diethyl ether. The ether fractions were dried over silica gel, and the volume condensed by passing dry nitrogen over the solution. The solution was next transferred to a 5-ml graduated cylinder, the silica gel washed twice with 0.5 ml portions of ether,, and the washings added to the bulk. Finally, the volume was adjusted to 5 ml and then transferred to a 5-ml rubber-stoppered syringe vial. One sample each of the BCl_3 and BF_3 treated solutions was also refluxed for

15 minutes. After this treatment, they were both treated in the manner described.

A separate sample of sesame oil was saponified in 0.5 M sodium hydroxide in methanol by refluxing for 1 hour at 65°C and then permitting the mixture to stand for 12 hours at room temperature. It was added to water and the unsaponifiable material extracted with petroleum ether. The water solution was acidified with 6 M hydrochloric acid and extracted again with petroleum ether. After evaporation of the petroleum ether under vacuum at room temperature, the free acids were dissolved in 5 ml of methanol and BCl_3 was bubbled through the solution for 30 seconds. The solution was then added to water, extracted with ether, dried over silica gel, the volume adjusted to 5 ml, and transferred to a syringe vial as with the preceding four samples.

Application to Lipids

Figure 4 shows the chromatograms obtained when equal portions of BCl_3 and BF_3 processed samples of sesame oil were injected into the gas chromatograph. A comparison of chromatograms A and B shows a slightly better yield for the transesterification technique than for the saponification method. It should be recalled that 15 second addition of BF_3 without refluxing will convert fatty acids to the esters. The relative proportions of oleic and linoleic acid are also slightly different. Apparently a small loss occurred during saponification or the saponification was incomplete. Chromatogram C shows the considerably lower yield obtained under equivalent transesterification conditions with BF_3. It should be pointed out that quantitative transesterification can be obtained with both reagents; however, BF_3 requires a somewhat longer refluxing period.

Tests with other natural lipid materials, such as butter, lecithin, and beeswax, were successful, showing that the BCl_3 transesterification method is applicable to a variety of materials.

Methylation of Acids

A test to compare the reaction time for free acids was conducted as follows: A standard solution of oleic acid was prepared by dissolving 5.102 grams of oleic acid in methanol to a volume to 50 ml. Eight 5-ml portions were transferred to reaction flasks. Boron trifluoride gas was bubbled into one 5-ml portion for ten minutes and then extracted as usual. Similar tests were performed in which BF_3 gas was introduced for two minutes, 30 seconds, and 15 seconds, respectively. These four tests were repeated with BCl_3.

In the comparison of the speed of methylation with BCl_3 versus BF_3, all eight tests gave the same chromatographic response. The conclusion

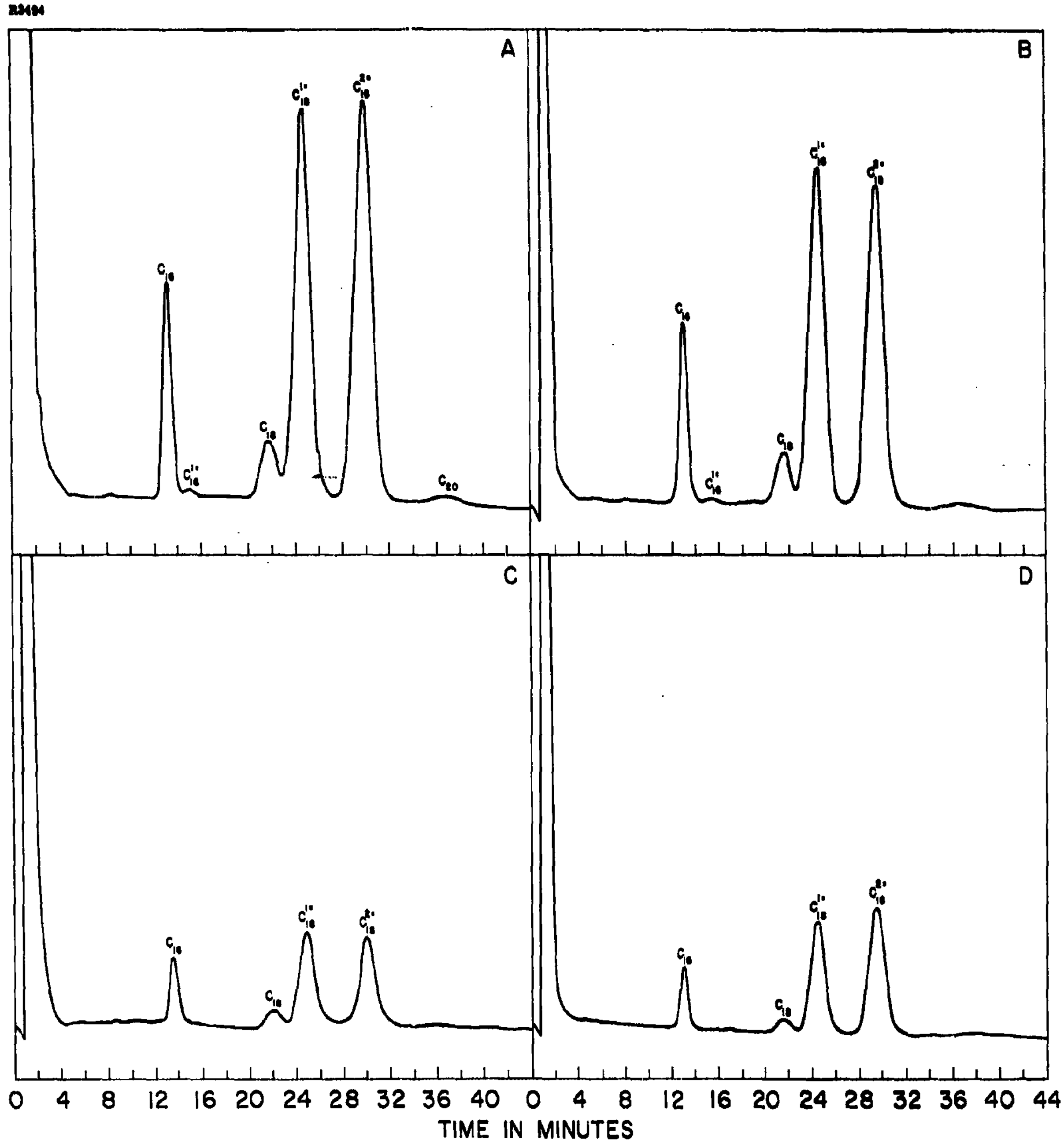

Figure 4. Comparison of BCl_3 and BF_3 on Sesame Oil

Each chromatogram represents 0.51 mg. sesame oil. Column is 6-foot by 1/4-inch OD copper containing 20-percent diethylene glycol succinate on type P Chromosorb, 60/80 mesh. Column isothermal at 225°C. Helium carrier flow rate is 100 ml/min

A. Bel_3 added for 4 minutes, followed by 15 minutes refluxing.
B. Saponified, Bel_3 added for 30 seconds, no refluxing.
C. BF_3 added for 4 minutes followed by 15 minutes refluxing.
D. Bel_3 added for 4 minutes with no refluxing.
BF_3 addition for 4 minutes with no refluxing yielded no methyl esters.

is that both gases are very efficient catalysts for esterification of the free fatty acid; they lead to complete esterification in 15 seconds.

Effect on Acid Structure

To test the possibility that BCl_3 treatment might alter the structure of an acid molecule, excessive treatment was applied to both a saturated acid (stearic acid) and an unsaturated acid containing two double bonds (linoleic acid). If BCl_3 causes conversion of unsaturated to saturated compounds, polymerization, or alkylation, it is to be expected that these changes would show up on the chromatograms resulting from excessive BCl_3 treatment. In both cases, the acids were dissolved in methanol, equal portions pipetted into volumetric flasks, and methanol added to make a 5-ml solution. Boron trichloride was then bubbled through the solution (with a reflux condenser attached) for various lengths of time and for various refluxing times.

Excessive BCl_3 treatment of saturated fatty acids did not result in any decrease or increase in yield of the methyl esters. Excessive treatment of an unsaturated fatty acid did, however, result in a decrease in yield of methyl ester, but no change in retention time resulted, nor was appreciable conversion to the saturated ester observed. Boron trichloride evidently catalyzed the formation of high-boiling polymers through double-bond polymerization; the polymers so produced were not eluted from the column. Apparently, the loss noted during treatment of the acid does not occur during treatment of the corresponding triglyceride. It can be concluded that under the conditions used for transesterification, no significant change of structure occurs.

Application to Bacteria

Lipid extraction from bacteria was conducted with a Soxhlet apparatus, using chloroform as the solvent. Various periods of reflux time were used. The chloroform was removed by evaporation with dry nitrogen before direct transesterification.

The following procedure was evolved for the direct extraction and transesterification of bacterial lipids, using the apparatus shown in figure 5.

a. About 1/2 gram of bacteria was weighed and transferred to a 25-ml volumetric flask; 10 ml of methanol and a glass-covered magnetic stirrer were added.

b. The reflux condenser was attached.

c. The assembly was placed on the hot plate-stirrer and the stirring rate adjusted to prevent both localized overheating and settling of the bacteria.

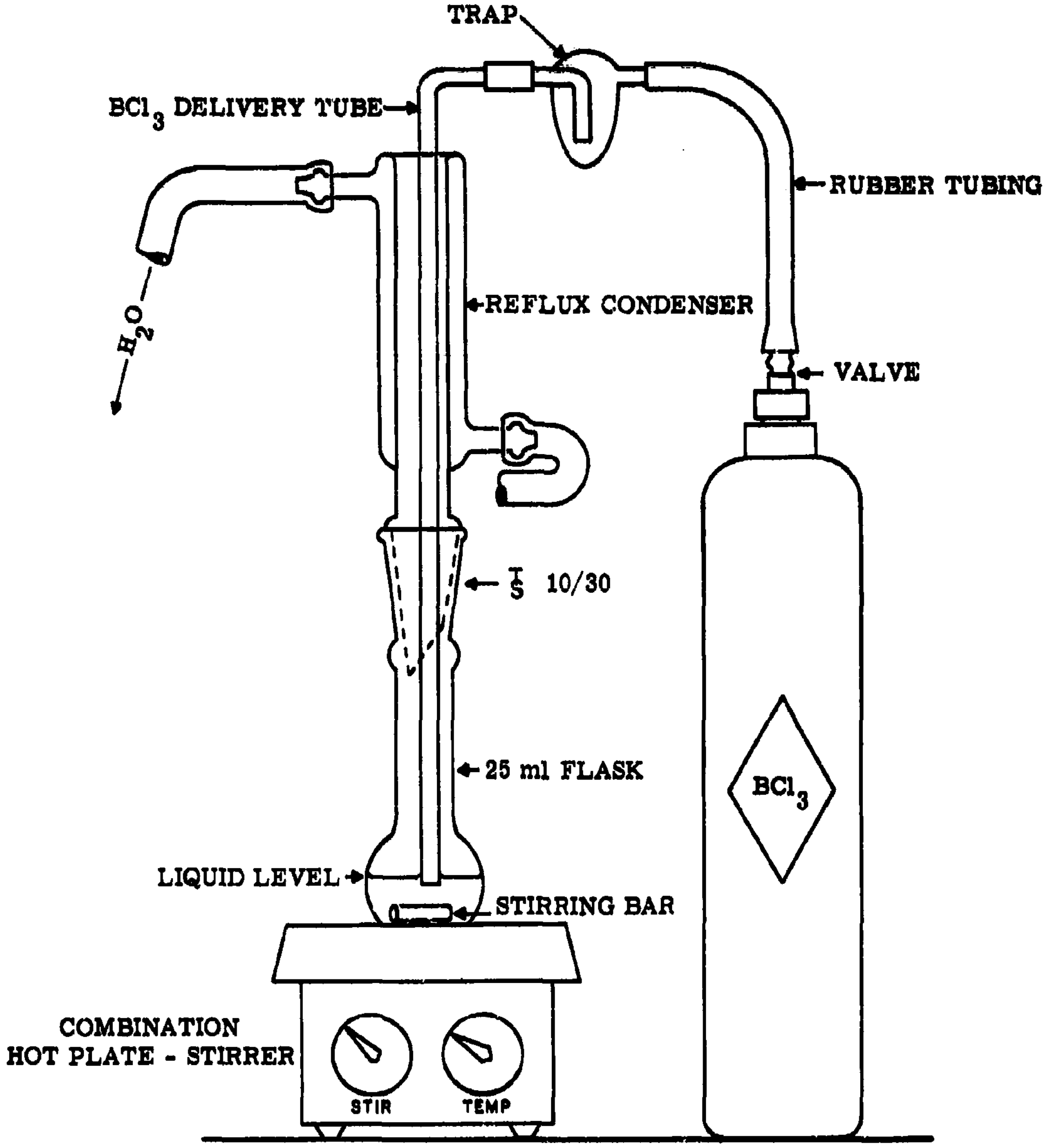

Figure 5. Apparatus for Direct Extraction-Transesterification of Bacteria

d. The BCl_3 delivery tube was inserted, with gas already passing through it at a rate of one gram of BCl_3 in two minutes. Although in practice the tip of the gas delivery tube was placed about 1 mm below the surface of the suspension, the gas delivery tube could remain above the suspension during delivery, provided stirring was vigorous.

e. In about one minute, refluxing began from the reaction of BCl_3 with the solution. The hot plate was then adjusted to maintain the required temperature for refluxing. At the end of two minutes, the BCl_3 delivery tube was removed from the solution and the has shut off.

f. The solution was refluxed for ten minutes.

g. The flask contents were transferred to a separatory funnel containing about 75 ml of distilled water.

h. The methyl esters were extracted with a 10-ml portion and two 5-ml portions of diethyl ether, using centrifugation to aid in the extraction.

i. The ether solution was dried by the addition of 3 to 5 grams of silica gel and the volume reduced by the use of dry nitrogen. This treatment was necessary when using the thermal conductivity detector, which is sensitive to water. On the columns that were used, water trailed very badly and disturbed the pattern of the chromatograms.

j. The solution was then transferred to a 5-ml graduated cylinder with ether washings of the silica gel. Actually, after two washings of the silica gel with ether, no detectable additional methyl esters could be removed from the silica gel. A test showed that no loss of esters occurred by treatment with silica gel.

k. The final volume was adjusted to the desired volume (usually about 2 ml) with ether and transferred to 5-ml syringe vials containing about 1/4 gram of silica gel.

The samples were then injected into the gas chromatograph for analysis. The samples injected contained an amount of esters equivalent to about 25-50 milligrams of original dry bacteria.

This procedure represents a convenient and rapid means for conducting studies of microbial composition. Figure 6 shows a comparison of the transesterification technique using a direct suspension of the bacteria in methanol and the technique involving Soxhlet extractions. It will be noted that different proportions of fatty acid methyl esters result from the two different methods. Furthermore, in this regard, the use of ethanol instead of methanol (which forms the ethyl esters rather than the methyl esters) also results in different proportions of the fatty

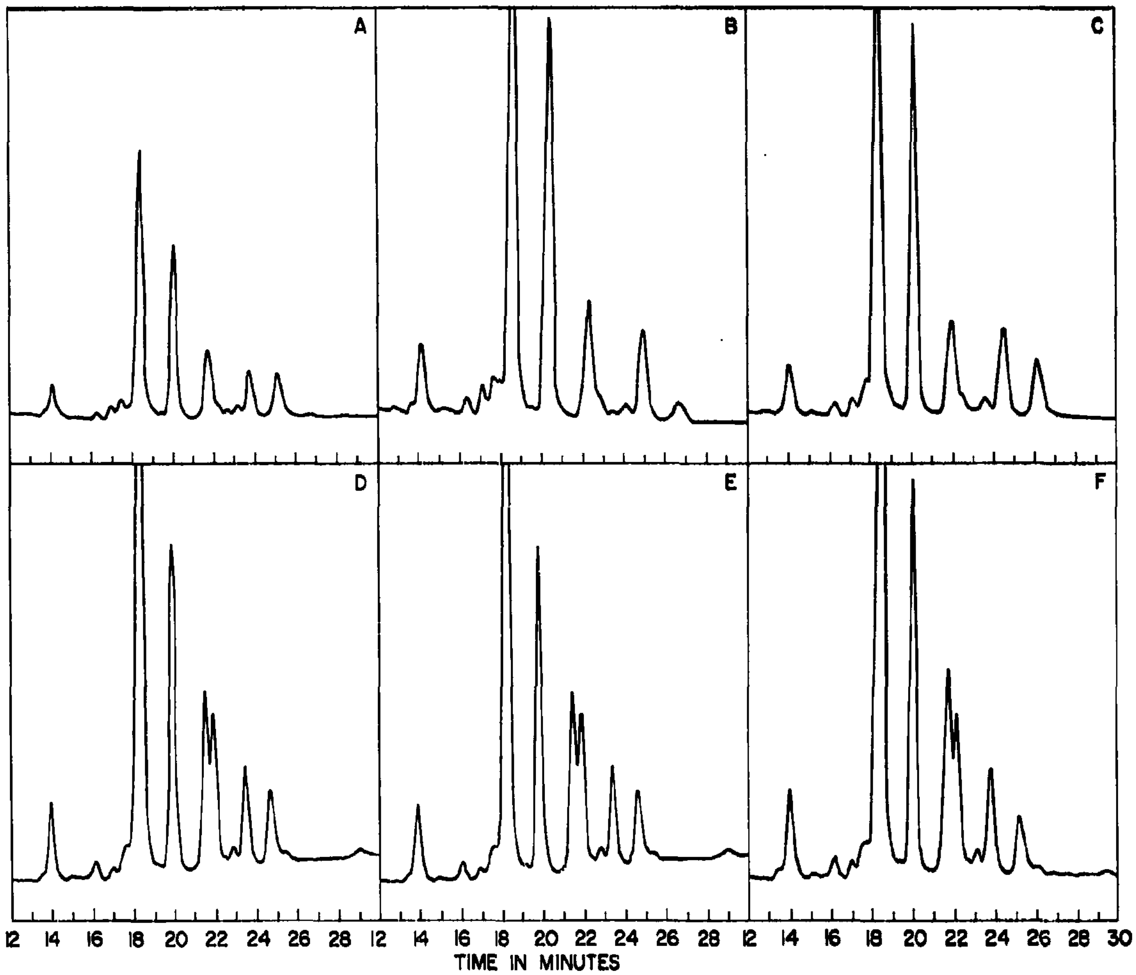

Figure 6. Methanol-BCl_3 vs. Soxhlet Extraction

Conditions: column is 7 feet by 1/4 inch, 10% SR, 1% Sebacic acid on Chromosorb Type W, 100/120 mesh, temperature programmed from 125° to 300°C at 5.6°/minute. Flow rate of helium is 100 ml/minute. The first 12 minutes of each chromatogram is not shown. Reference marks are at 1-minute intervals. Each chromatogram represents 50 mg S. marcescens.

A. BCl_3 gas added to methanol, 20 minutes refluxing.
B. BCl_3 gas added to methanol, 10 minutes refluxing.
C. BCl_3 gas added to methanol, 5 minutes refluxing.
D. 180 minute Soxhlet extraction with chloroform followed by transesterification.
E. 90 minute Soxhlet extraction with chloroform followed by transesterification.
F. 45 minute Soxhlet extration with chloroform followed by transesterification.

acid esters than with either direct methanol suspension or Soxhlet extraction. (See figure 7).

Because of the results shown in the two preceding figures, an attempt was made to add a good solvent for fats to the methanol suspensions of bacteria to aid in the extraction of lipids. Diethyl ether was used in an amount equal in volume to the methanol used. For Serratia marcescens only about 25 percent as much extraction-transesterification occurred for this solvent mixture as when methanol alone was used. Similar results were obtained with Pasteurella tularensis. Chromatograms representing 100 mg of each organism following this treatment are shown in figure 8. It will be noted by comparison with a standard mixture of methyl esters, that resolution was very poor also. Base line behavior was also indicative of the presence of unesterified lipids or fatty acids.

As mentioned previously, lithium methoxide has been used to achieve rapid transesterification. Attempts to apply this method directly to bacterial suspensions were only partially successful, although we did find that it is an effective transesterification agent for sesame oil. This is undoubtedly caused by the fact that the reagent does not extract the lipids from the bacteria. In this regard, mixtures of ether and methanol were used successfully in the transesterification of sesame oil by BCl_3, while similar attempts using cyclohexane in methanol produced only a very limited amount of transesterification. Accordingly, it is concluded that the addition of these solvents interferes with, rather than aids, the extraction-transesterification by BCl_3. The manner of interference is not clearly understood and more work should be done in connection with this point.

As far as we can determine, boron trifluoride has not been applied to transesterification by previous investigators. The present work shows that this reagent, as well as boron trichloride, can be used for direct methylation of lipids. In addition, the boron-trichloride procedure allows a direct conversion and extraction of fatty materials from bacteria. Boron trichloride is superior to boron trifluoride in the rate of conversion in this application as well as in its extraction effectiveness.

The use of boron trichloride has other practical advantages also. Boron trichloride gas does not combine with methanol at as rapid a rate as boron trifluoride. As a consequence, the addition of the gas through a bubbler tube is more easily controlled without danger of the methanol being sucked up the tube. It is not necessary to cool the methanol before addition of the reagent. The heat of reaction provides the temperature necessary to start moderate refluxing immediately, and the solvent effect of the reaction mixture allows direct extraction of bacterial lipids. Because of the dehydration effect of boron trichloride, the direct extraction and transesterification of bacteria does not require drying or

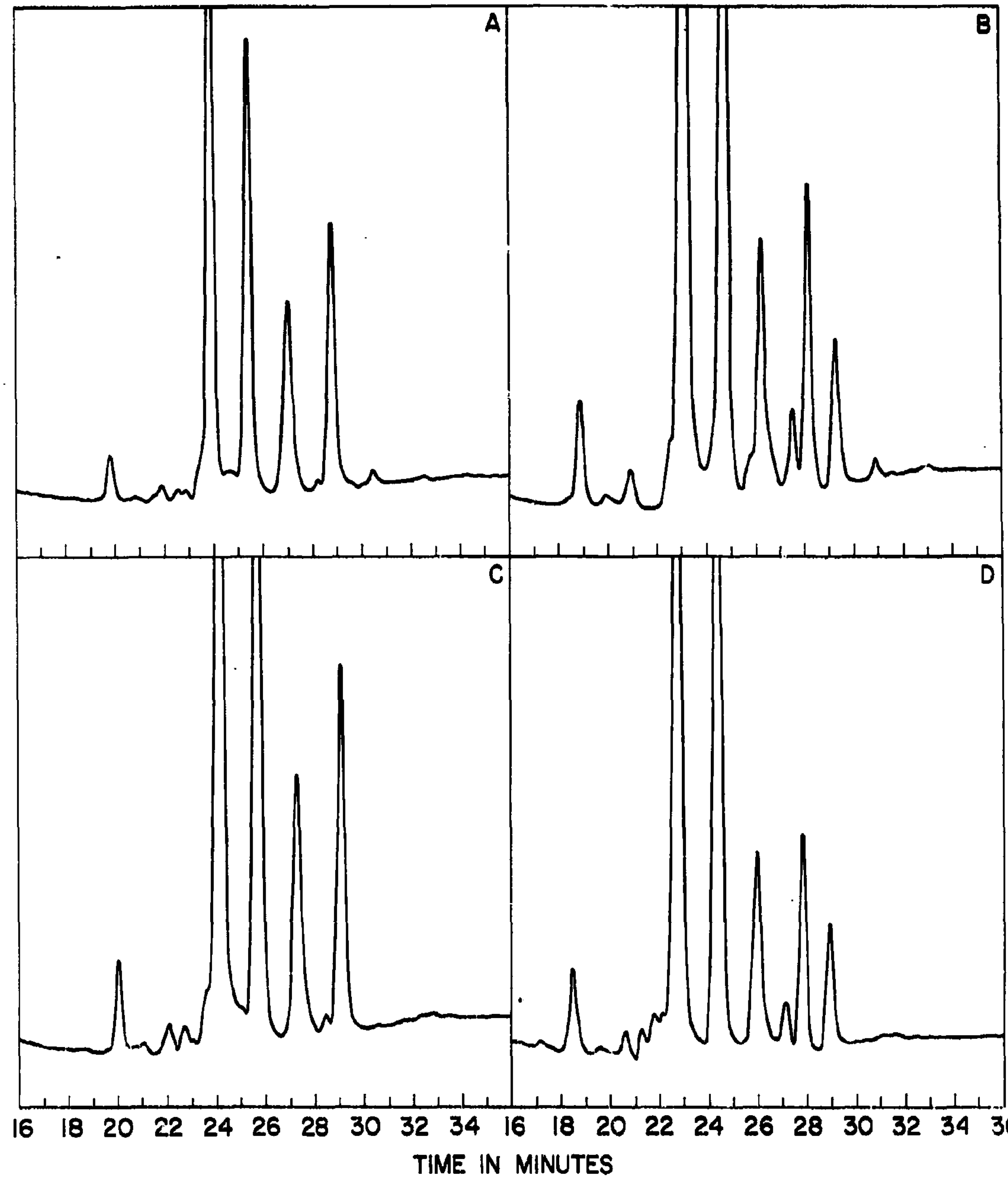

Figure 7. BCl_3 gas vs. BCl_3-ethyl Ether Extraction and Transesterification

Conditions and Column same as Figure 6.
A. 8 ml ethanol + 8 ml BCl_3-ethyl ether, 5 minute reflux
B. 8 ml methanol + 8 ml BCl_3-ethyl ether, 5 minute reflux.
C. 16 ml ethanol, 2 minutes BCl_3 gas addition, 5 minute reflux.
D. 16 ml methanol, 2 minutes BCl_3 gas addition, 5 minute reflux.

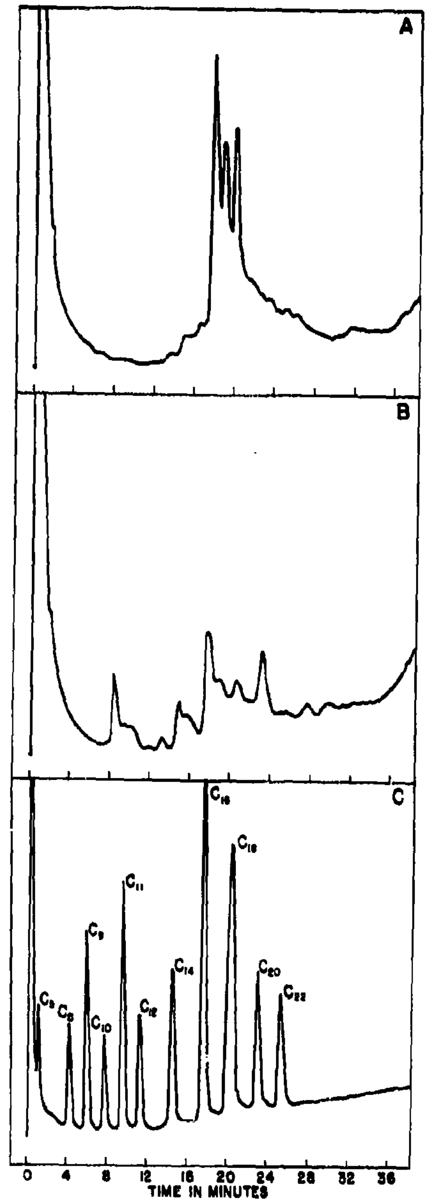

Figure 8. Transesterification of Bacteria by BCl_3 Using a Mixture of Ether and Methanol

Conditions: 2 ft x 1/4'' OD column, 5% SR on Anakrom ABS. Temperature programmed from 125°-390° at 7.9°/min.

A. Serratia marcescens, representing 100 mg.
B. Pasturella tulerensis, representing 100 mg.
C. Comparison with mixture of methyl esters.

lypohilization; it works as well with wet, freshly centrifuged bacteria as with the dry bacteria.

Comparison of Extraction Effectiveness

It was noted that different solvents (methanol, ethanol, or chloroform with the Soxhlet extraction) lead to different yields of certain fatty acid methyl esters. The greatest overall yield occurs with chloroform as the solvent; this yield variation is undoubtedly caused by differences in solubilities of specific fatty acids or lipids in the various solvent systems. However, it is apparent that the short methanol-BCl_3 extraction-transesterification procedure is as adequate for detection or identification purposes as the prolonged Soxhlet extraction approach with chloroform. Chloroform in the direct BCl_3 method forms a gelatinous compound of unknown composition; consequently, it must be removed before transesterification.

The advantage of using a prepared liquid reagent, such as the BF_3-methanol complex of Metcalfe,[12] was recognized. The following tests were made with this in mind. Boron trichloride was bubbled into methanol and into diethyl ether to form the BCl_3-solvent complex.

Methanol saturated with BCl_3 was used in place of BCl_3 gas reagent. The results are shown in figure 9.

In diethyl ether a solid complex is formed when BCl_3 is added to the ether. This complex is soluble in excess ether. The complex was diluted with ether in realizing complete solubilization. About five times as much ether as BCl_3 by weight was used. This diluted reagent was used in place of BCl_3 gas addition.

A comparison of the effect of this ether complex when added to methanol and ethanol suspensions of bacteria with the addition of the gas directly to methanol is shown in figure 10. Two minutes of bubbling BCl_3 add slightly more than one gram of BCl_3 to the reaction mixture. Sufficient BCl_3-ethyl ether was added to duplicate suspensions representing two grams of BCl_3. The results indicate that the ether complex leads to the same transesterification as does the direct addition of gas. The reactive nature of even the dilute ether complex is such, however, that it must be slowly added to the methanol suspension over a period of approximately one minute to avoid violent reaction.

The amount of transesterification is proportional to the amount of BCl_3 added to suspensions up to the point where an amount of BCl_3 is added which leads to the complete removal of water from the mixture and provides a slight excess of BCl_3 as a catalyst. Additional BCl_3 then has no effect. Excess BCl_3 beyond the proportional region may result in unwanted side reactions, particularly with unsaturated acids. The presence of water causes a shifting of the equilibrium to favor the fatty acids rather than the methyl esters. With insufficient BCl_3, completely anhydrous conditions are not attained, and a lower yield of methyl esters is observed. The BCl_3 ether complex does not retain its activity at room temperature. Storage for 4 weeks at 25°resulted in a 50% loss in activity.

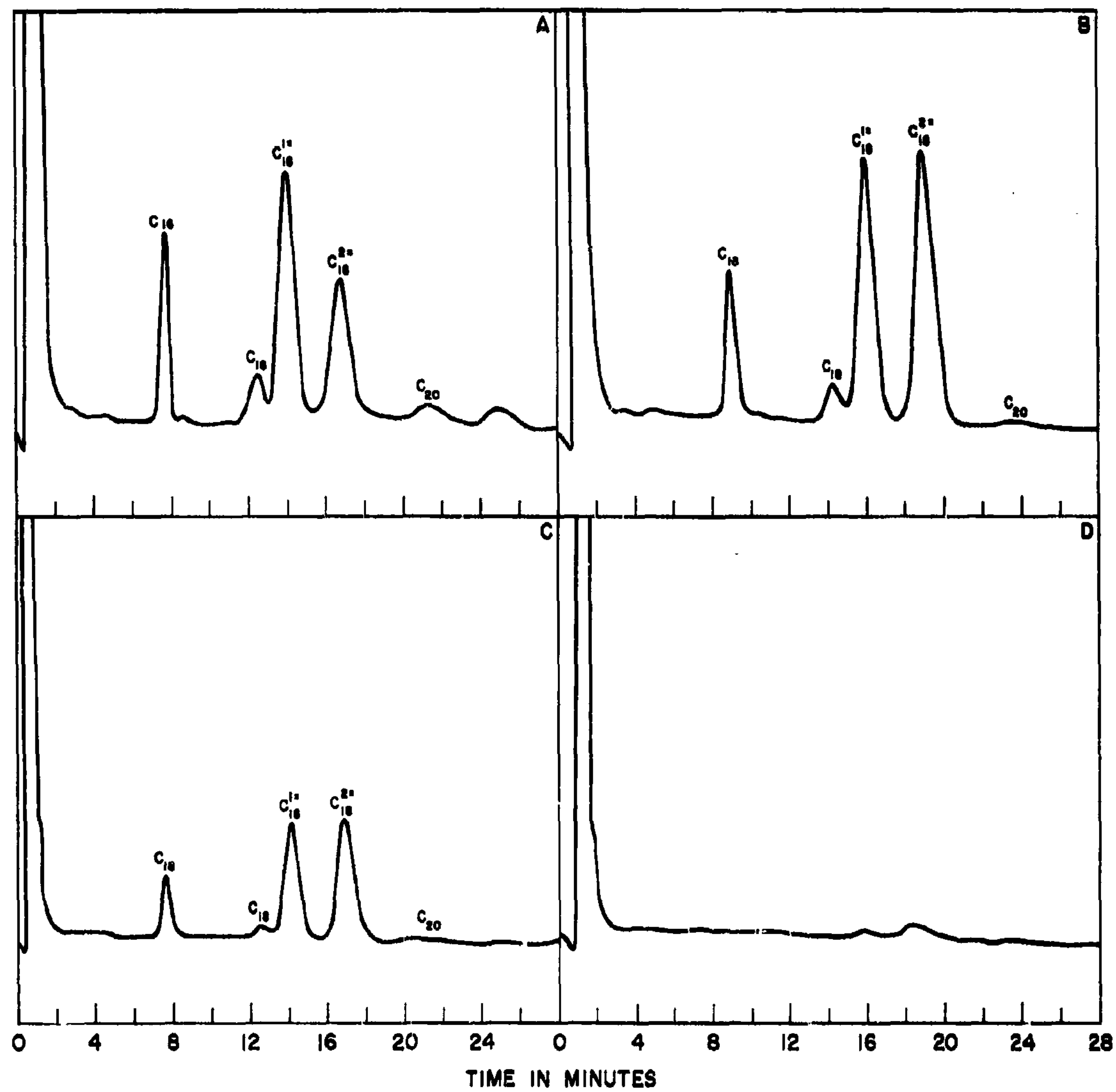

Figure 9. Transesterification with BCl_3 Pretreated Methanol

Each chromatogram represents 0.51 mg. sesame oil.
Column and conditions the same as in figure 10.
A. BCl_3 for 8 minutes with continuous refluxing.
B. Methanol saturated with BCl_3, 15 minutes refluxing.
C. Methanol saturated with BCl_3, 5 minutes refluxing.
D. Methanol saturated with BCl_3, no refluxing.

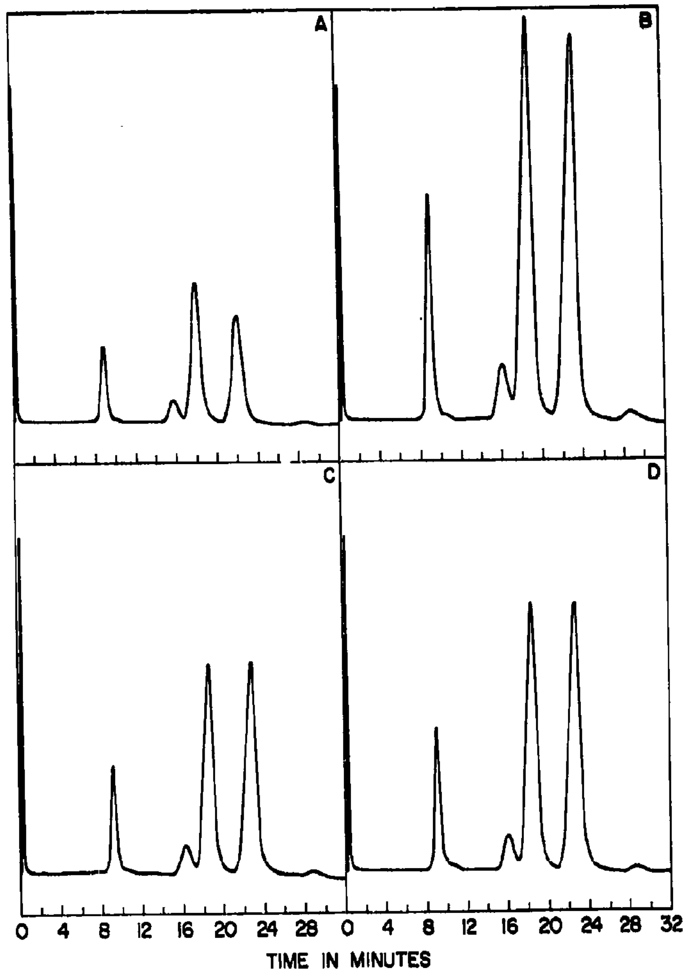

Figure 10. BCl_3 gas vs. BCl_3-ethyl Ether Transesterification

Conditions: 6-foot x 1/4-inch OD column, 20% DEGS on Type P Chromosorb, 60/80 mesh, isothermal operation at 225°C. Helium carrier gas flow rate is 100 ml/min. Each chromatogram represents 1.36 mg sesame oil. Reference marks every 2 minutes. Ether peak attenuated 1/1024.

A. 4 ml ether, 6 ml methanol, 4 minutes BCl_3 gas addition, no refluxing.

B. 4 ml ether, 6 ml methanol, 4 minutes BCl_3 gas addition, 15 min. refluxing

C. 4 ml ether, 6 ml methanol, 2 ml BCl_3-ether reagent, no refluxing.

D. 4 ml ether, 6 ml methanol, 2 ml BCl_3-ether reagent, 15 min. refluxing.

These results indicate that BCl_3 gas is preferred for the transesterification because: (a) the gas is easily handled, (b) the addition of the gas involves less hazard than addition of the ether complex, and (c) the ether-complex cannot be stored at room temperature. Furthermore, since the amount of BCl_3 required is proportional to both the amount of methanol used in the extraction-transesterification and to the gas delivery time, decreasing the amount of methanol will shorten the length of bubbling time proportionally. In all cases methanol is required in relatively large excess compared to the lipids extracted.

Because BCl_3 reaction with methanol goes beyond the formation of a coordination compound, the preparation of the BCl_3-methanol mixture is not effective. Methanol saturated with BCl_3 was found to produce a limited lipid transesterfication; its effectiveness is quite low when compared with gaseous BCl_3. The direct addition of liquid BCl_3 to cooled methanol is not suitable because violent eruption and ignition of the methanol occurs.

3.2 Bacterial Work

3.2.1 Growth Technique

Seven nonpathogenic bacteria were obtained from the American Type culture Collection, 2112 M Street, N. W., Washington, D. C. These were Micrococcus ureae, Gaffkya tetragena, Escherichia freundii, Aerobacter cloacae, Klebsiella aerogenes, Proteus vulgaris, and Escherichia coli. These were grown by the technique to be described. Four additional bacteria, Serratia marcescens, Pasterurella tularensis, Bacillus subtilis, var. niger, and Bacillus anthracis, were obtained in dried form from the U.S. Army Chemical Corps, Biological Laboratories, Ft. Detrick, Md. The latter two were in the spore form. The growth media for these is not known. For the others, the culture media used was either Trypticase Soy Broty (TSB) or Koser Citrate media (KCM), obtained from Baltimore Biological Laboratories, Baltimore, Md. These were chosen because they were free of lipids. The TSB was analyzed for lipid content; and this content was found to be less than 0.2 percent. The KCM is a totally synthetic media containing only citrate as a source of carbon.

The bacteria were grown in the apparatus shown in figure 11. It consists of a 2.8 liter culture flask to which has been added a 25 watt or 50 watt aquarium-type tube immersion heater capable of controlling the temperature within $\pm 1^{\circ}C$, an air-bubbling tube, and a port with a serum cap for sterile injection and removal of bacterial samples. The flask is capped with an inverted beaker. Millipore filters were used to sterilize the air supplied by the laboratory air line. Sterilization was effected either by conventional autoclaving, or by adding liquid ethylene oxide (bd $10.7^{\circ}C$) at the rate of 5 ml. per liter of nutrient and allowing it to sit undisturbed for two to four hours at room temperature, followed by overnight bubbling with 20% ethylene oxide, 80% carbon dioxide at incubation temperatures. This step was then followed by aeration for 48 hours by bubbling air through the nutrient solution. When conventional autoclaving was used, the air lines were sterilized before and during the connection by a flow of the ethylene oxide-carbon dioxide gas mixture.

R3494

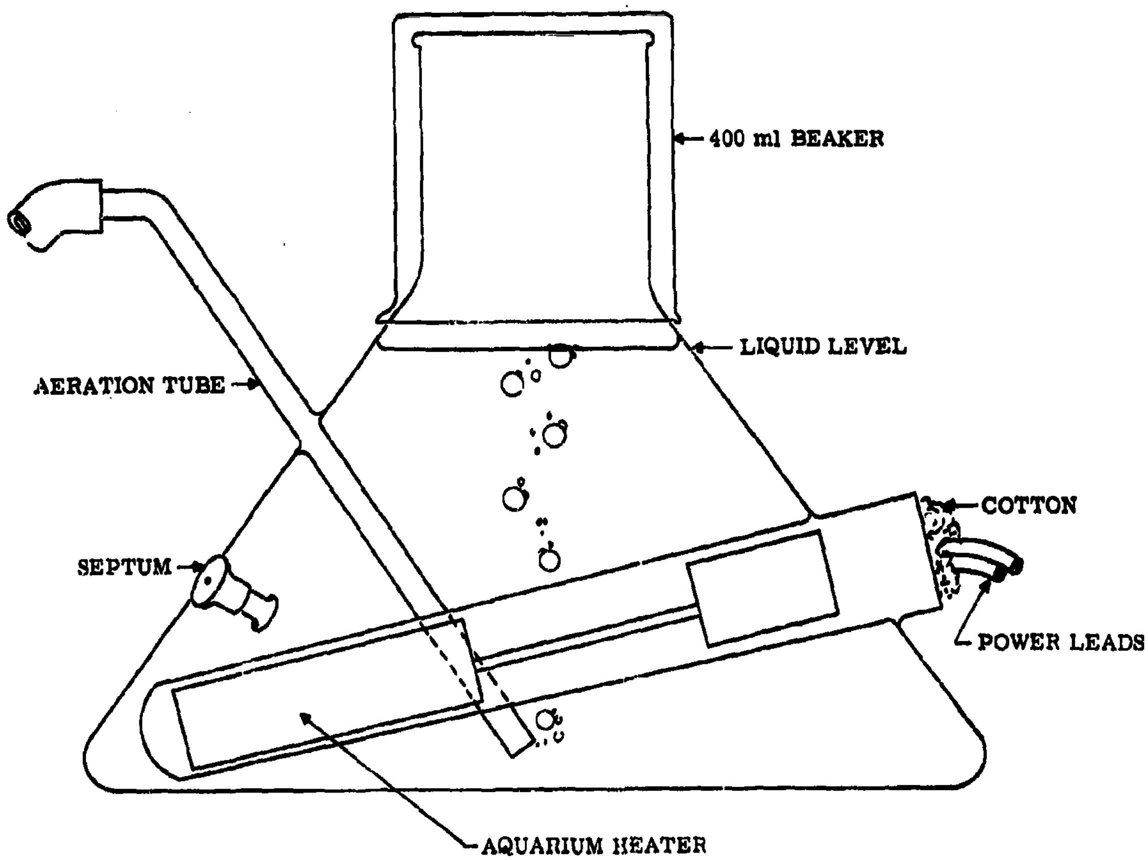

Figure 11. Culture Flask for Growing Small Batches of Bacteria

After establishing the sterility of the nutrient solutions by observing no growth during 48 hours of air bubbling, pure cultures obtained from the American Type Culture Collection were added to the nutrients. The cultures could then be maintained from flask to flask by extraction and injection with sterile syringes.

After the growth of the bacteria was completed, the culture was centrifuged at 4000 g for 45 minutes. The sedimented bacteria were resuspended in 1 percent solution KCl and recentrifuged. The bacteria were then taken up in a small amount of distilled water, frozen in a dry ice-acetone bath, and lyophilized. Temporary smears obtained during the growth were examined under the microscope to determine the appropriate morphological characteristics and the purity and identity of the culture. No further tests were made to verify the identity of the bacteria.

One flask (about 2.5 liters) of nutrient would yield up to 3 gms of bacteria (dry weight) for TSB and up to 1 gram for KCM.

3.2.2 Chromatographic Patterns Obtained From Bacteria

Bacterial lipid chromatograms, grouped to shown the similarities between family members, are shown in figure 12; the differences between families in figure 13, and the differences between individual species grown on two radically different culture media are shown in figure 14. Two growth forms of Klebsiella aerogenes are shown in figure 15. The Klebsiella aerogenes was induced to grow in a modified form (filiform) through the incomplete removal of ethylene oxide from the nutrient, as well as in its normal encapsulated form.

Figure 16 shows a comparison of the patterns obtained from Escherichia coli at different growth stages.

The Escherichia coli were harvested about midway through the logarithmic growth phase, near its end, and two days after its end to compare the lipid patterns of each growth period.

Apparently, the amount of unsaturated C_{16} lipid decreases and that of saturated C_{16} lipid increases as the growth period increases. Also, the C_{18}, C_{19}, and C_{20} lipids undergo a change in quantity. Another significant change, noted from the quantity of bacteria required for each chromatogram, is that the bacteria harvested earlier have a much higher lipid content. The qualitative pattern type remains the same throughout the growth period, and microscopic observation shows no change in morphology.

It becomes evident that each species has a unique pattern if grown on similar nutrient media. The pattern type remains the same regardless of nutrient media, but the relative amounts of each lipid in the bacteria differ with differing media and with different growth stages. The chromatograms also suggest that each family has a distinctive pattern type which can be recognized without difficulty. The exception to this is the case of the Bacillaceae members subtilis and

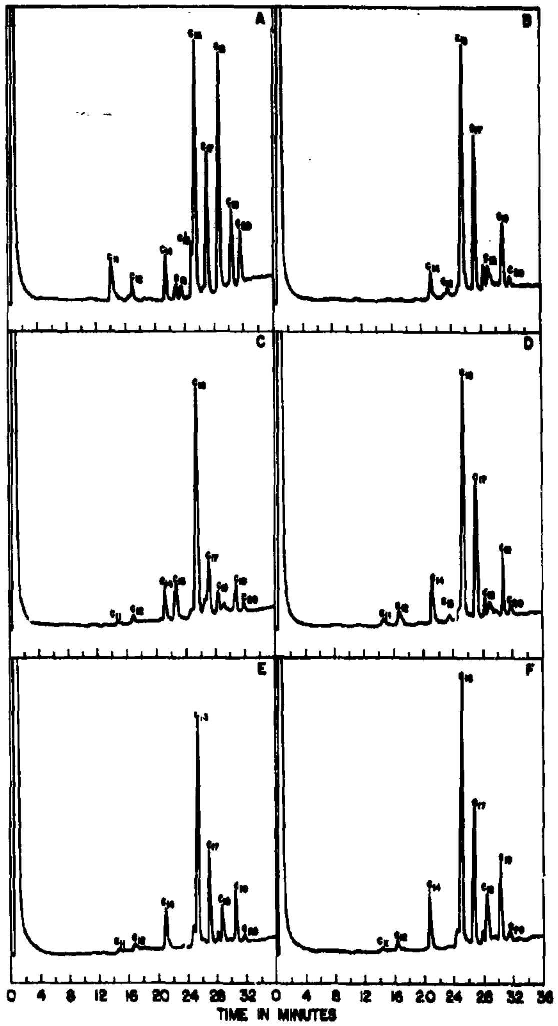

Figure 12. Comparison of Genus Differences in Family Enterobacteriaceae

Each chromatogram represents 25 mg. of bacteria, dry weight. Column is 16 feet x 1/4 inch OD copper containing 3-percent Apiezon W on Anakrom Type ABS 160/170 mesh. Column temperature programmed from 125° to 300°C at 5.6°C/min. Fatty acid chain lengths corresponding to normal acids are shown by subscripts.

A. Escherichia coli grown in TSB.
B. Serratia marcescens grown in unknown nutrient media.
C. Proteus vulgaris grown in TSB.
D. Escherichia freundii grown in TSB.
E. Aerobacter cloacae grown in TSB.
F. Klebsiella aerogenes grown in TSB.

R4311

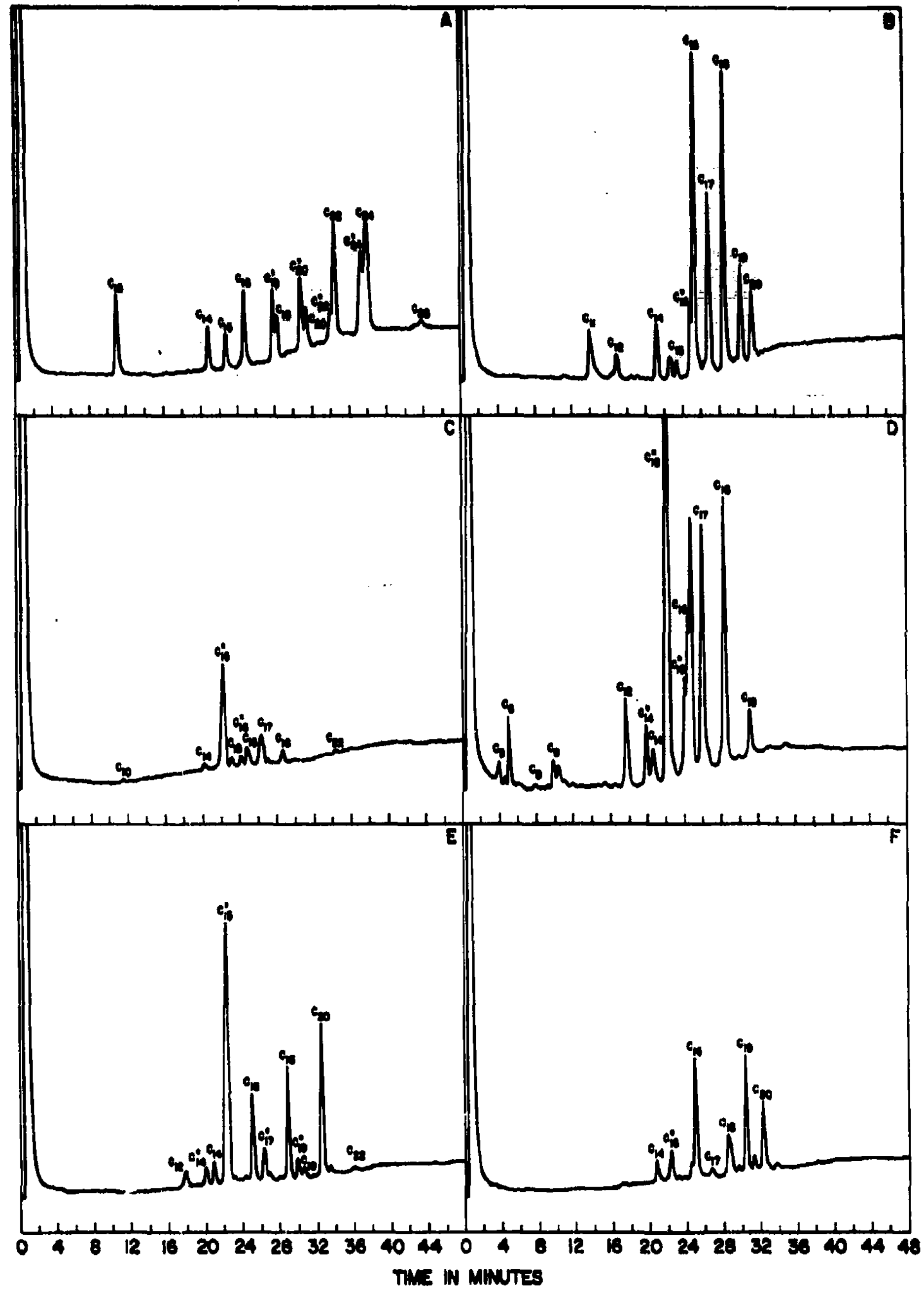

Figure 13. Comparison of Family Differences in Class Schizomycetes

Conditions and Column same as Figure 12.

A. Parvobacteriaceae, Pasteurella tularensis, nutrient unknown.
B. Enterobacteriaceae, Escherichia coli grown in TSB.
C. Bacillaceae, spore form of Bacillus subtilis nutrient unknown.
D. Bacillaceae, spore form of Bacillus anthracis, nutrient unknown.
E. Micrococcaceae, Gaffkya tetragena grown in TSB.
F. Micrococcaceae, Micrococcus ureae grown in TSB.

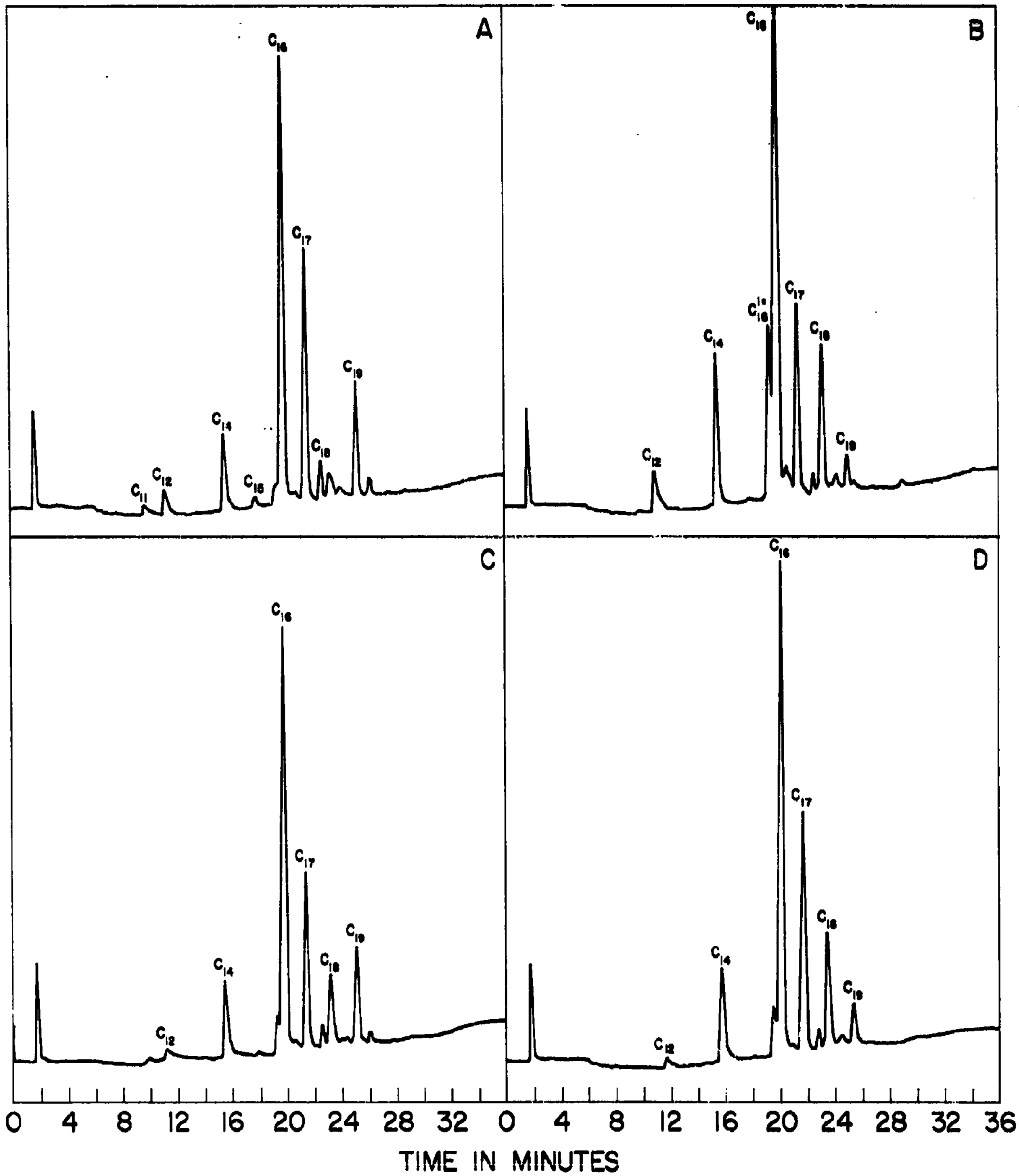

Figure 14. Comparison of Growth Media Effects

Conditions and Column same as Figure 12.

A. Escherichia freundii grown in TSB.
B. Escherichia freundii grown in KCM.
C. Aerobacter cloacae grown in TSB.
D. Aerobacter cloacae grown in KCM.

R3484

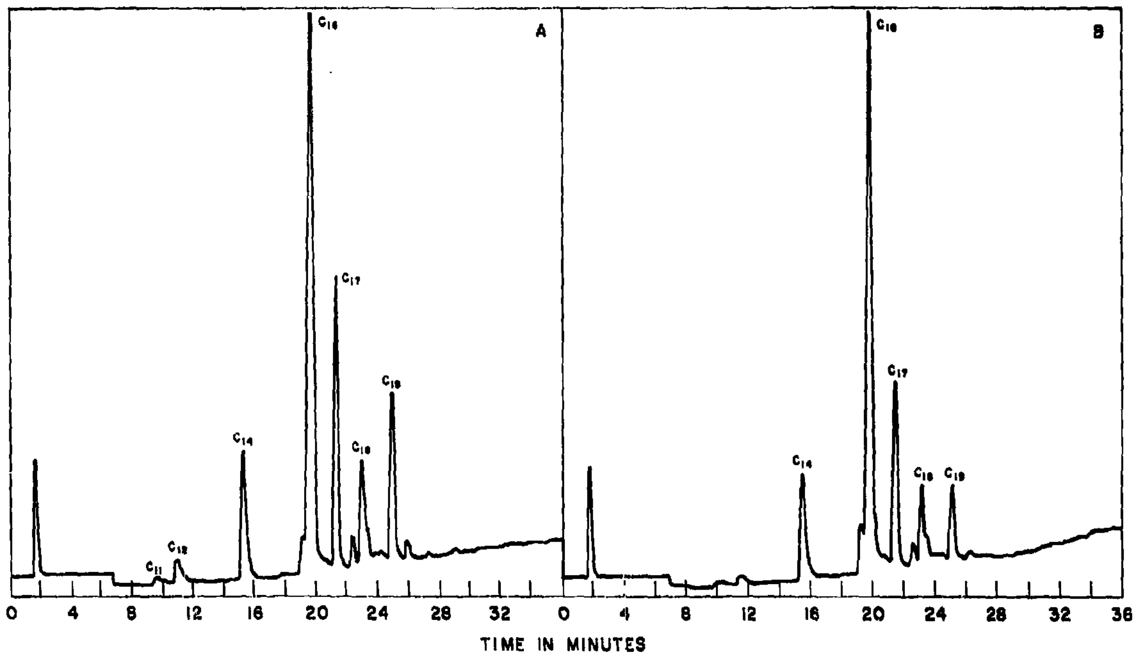

Figure 15. Comparison of Growth Form Effects

Conditions and Column same as Figure 12. Ether peak attenuated 1/1024.
A. Klebsiella aerogenes, normal capsular growth.
B. Klebsiella aerogenes filiform growth. Both grown in TSB.

H4312

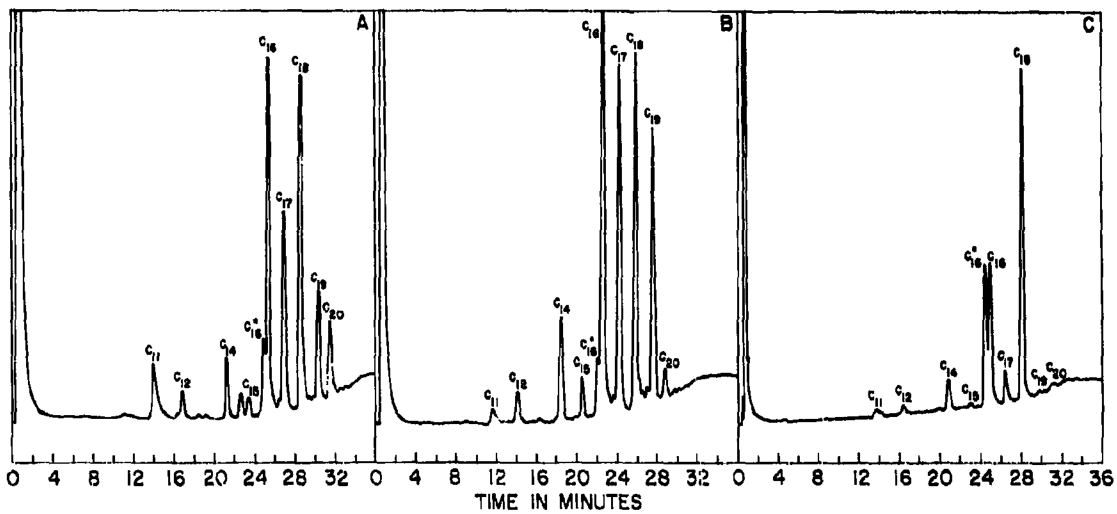

Figure 16. Comparison of Pattern for Different Growth Periods of Escherichia Coli

Conditions and Column same as Figure 12.
A. Bacteria left in nutrient at incubation temperature for 2 days after completion of log-growth phase. Chromatogram represents 80-mg. bacteria dry weight.
B. Growth interrupted near completion of log-growth phase. Chromatogram represents 33-mg. bacteria, dry weight.
C. Growth interrupted half way through log-growth phase. Chromatogram represents 7-mg. bacteria, dry weight.

anthracis, whose patterns are not closely similar. This may be because only the spore forms were used.

It has been shown by Anderson[6] on the basis of chemical studies on several strains of bacillus that each strain produces a lipid composition peculiar to itself, and that although the compositions are not identical similarities between strains are shown. The work reported here further indicates the appearance of family resemblances. It also establishes the validity of the concept of the concentration profile.

3.3 Spectrophotometric Methods

3.3.1 Introduction

A completely separate approach to the concentration profile technique is the investigation of spectrophotometric methods for the sensitive and characteristic analysis of components of bacteria and spores. In general, this work has been of an exploratory nature in evaluating the possibilities of this approach.

3.3.2 Work Accomplished and Conclusions

Literature Survey:

Acid Hydrolysis of Nonprotein Constituents

A literature survey has revealed that short-time exposure to acid converts several constituents normally found in bacteria cells, cell walls, or spores into compounds which can directly, or through the action of additive reagents, be determined by spectrophotometric methods. These constituents are: (a) dipicolinic acid (DPA), (b) 3-Hydroxybutanoic acid (HBA), (c) nucleic acids, and (d) carbohydrates.

Dipicolinic acid, which appears to be unique to bacterial spores or spore-forming bacteria can be completely liberated by boiling in acid for 15 minutes.[17] Dipicolinic acid has the more-or-less typical ultraviolet absorbance spectra associated with pyridine and pyridine derivatives. This spectra shows an absorbance maximum at 270 mu under prescribed conditions with a molar absorptivity of 4.5×10^3 and is, therefore, capable of being used directly for microgram determinations.[18] In addition, the compound develops a yellow color when allowed to complex under suitable conditions with ferrous iron. However, the molar absorptivity of this method would only be 15 percent of the ultraviolet absorbance method.[19]

Poly-3-hydroxybutanoic acid, a polymer found in bacterial cell walls, is degraded to the monomer and then dehydrated to crotonic acid by ten minutes heating at 100°C in concentrated sulfuric acid.[20] Crotonic acid also has a unique ultraviolet absorbance spectrum in concentrated acid. The maximum absorbance occurs at 235 mu in 80-100 percent sulfuric acid and has a molar absorbance index of 1.55×10^4. This method would be sensitive to microgram quantities. Other methods for determining this polymer are the gravimetric method[21] and a turbidity method,[22] neither of which possess sensitivity of the ultraviolet absorbance method.

According to several reports, nucleic acids, when heated with hydrochloric acid at 100° for 30 to 60 minutes, liberate reproducibly pentoses; the pentoses are subsequently converted to furfural. Furfural has a unique absorption spectrum in the ultraviolet with a reported maximum at 278.5 mu. It is sufficiently volatile to be distilled with steam from the acid; distillation has been used for its collection and analysis.[23]

A simpler method involves the addition of one of several colored reagents to the nucleic acid solution. The nucleic acid-reagent reaction leads to a less volatile colored compound; the decrease in color intensity is measured colorimetrically or spectrophotometrically. Effective reagents of this type are: resorcinol, orcinol, phloroglucinol, aniline, xylidine, and benzidine, as well as certain derivatives of these compounds.[24-25] Several of these reagents react to give colored compounds which are insoluble in aqueous solutions, but which can be extracted and concentrated with suitable solvents such as isoamyl alcohol.[26] Others, like aniline, do not develop suitable color, except under defined conditions.[21] The nature of most of these reactions is not clearly understood, but these reactions are probably similar to the reactions of benzaldehyde in the production of dyes (for instance, the reaction of benzaldehyde in the presence of hot hydrochloric acid and zinc chloride with dimethylaniline gives Malachite green).[27]

Carbohydrates are hydrolyzed to simple sugars and sugar derivatives in less than 30 minutes by concentrated hydrochloric acid and heat and then converted to furfural derivatives.[28] Hexoses yield 5-(hydroxy-methyl)-furfural, while 6-deoxyaldehexoses yield 5-methylfurfural. These compounds are not volatile with steam as is furfural, and they react with several of the agents with which furfural reacts to yield compounds with visible and U.V. spectra differing significantly from those associated with furfural reaction products. For example, the absorbance maxima of the orcinol reaction product with pentoses in hydrochloric acid in the visible spectral region are at 670 mu and 450 mu, while the reaction product with hexoses gives peaks at 520 mμ and 450 mμ. Because of these differences, hexoses and pentoses may be determined simultaneously.[29] The molar absorbance indices of two hexose reaction products at 250 mμ are considerably lower than the molar absorbancy index for a typical pentose reaction product at 670 mμ. Some values, calculated from published absorption spectra of the reaction products of the following sugars are: fructose = 1×10^3, glucose = 2.2×10^2, and xylose = 3×10^3. These differences are probably caused by the fact that hexose continues to react in acid solution to form an oxidation product, levulinic acid.[28] The addition of the colorimetric reagent before heating may inhibit this latter oxidation.

Acid Hydrolysis of Protein Constituents

Complete hydrolysis of proteins to their constituent amino acids is generally accomplished by heating the protein with 5 to 20 times its weight of 3N to 12N hydrochloric acid at 100° to 120° for 3 to 40 hours.[30] In general the amino acids are stable under these conditions with the exception of tryptophan, cysteine, serine, and threonine. Partial hydrolysis begins immediately on exposure to acid (or base). There is a possibility of attaining a more rapid hydrolysis of proteins through the use of ion-exchange resins which catalyze the hydrolysis. For instance, Dowex-50 was found to be 115 times more effective than an equivalent amount of hydrochloric acid.[31] The hydrochloric acid concentration against which this comparison was made was 1N. No data were provided from which the resin catalyzed could be compared with that for concentrated acid. It is conceivable that more active catalysts can be found. It is of interest in this regard to note that certain bivalent cations catalyze the hydrolysis of ribonucleic acid.[32] There is a possibility that these same cations may speed the hydrolysis of proteins as well.

Experimental

Ultraviolet Absorbance of Dipicolinic Acid

Although dipicolinic acid has been extracted from spores by treatment with hot acid, no absorbance spectra have been reported for this material in the presence of acids. It has been found that dipicolinic acid has a unique absorption spectrum in concentrated hydrochloric acid with strong peaks at 215 mμ and 278 mμ (figure 17). Hydrochloric acid concentrations between 20 and 37 percent result in a progressive shift of the absorption maxima, but by no more than 2 mμ. Hydrochloric acid concentration near 27 percent affects the absorbance to the greatest extent and a 30 percent acid concentration leads to minimum absorption. The relative difference between maximum and minimum absorption at a particular wavelength is the order of 15 percent.

The absorbance of dipicolinic acid follows Beer's law over the range from 1×10^{-5} M to at least 1×10^{-4} M with molar absorbance indexes of 8.22×10^3 at 278 mμ and 9.6×10^3 at 215 mμ in 37 percent hydrochloric acid. The absorbance at 215 mμ is affected by slight variations in effective hydrochloric acid concentration which might exist between the reference and sample, and it is not as reliable for quantative determinations. Heating to one hour has no effect on the absorption spectrum.

For the studies on dipicolinic acid, a commercially obtained product was recrystallized from boiling water, dried under vacuum. Weighed quantities were dissolved in concentrated hydrochloric acid and then diluted to the desired concentrations with concentrated hydrochloric acid. For studies on the effect of heating of dipicolinic acid (and all the materials reported below), the desired concentration was made up in a 100 ml volumetric flask; the flask was immersed in a boiling water bath. Samples were withdrawn using a 5 ml pipette

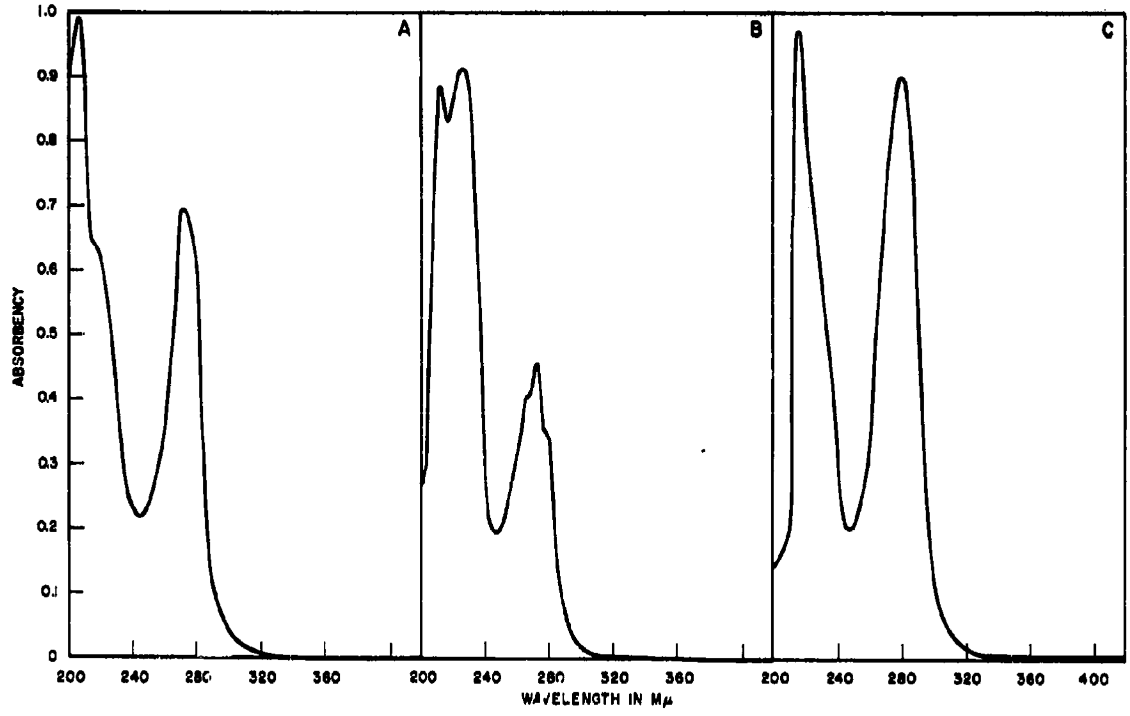

Figure 17. Absorption Spectra of Dipicolinic Acid

Concentration 0.020 mg/ml., 1 cm. path length cells.
Solvent: A. distilled water
B. 95% ethanol
C. Concentrated HCl

from the flask at 5-, 10-, or 60-minute intervals, depending upon the study involved. The sample was cooled to room temperature and its spectrum was recorded with the B & L Spectronic 505 using concentrated hydrochloric acid in the reference beam.

In the studies on the effect of hydrochloric acid concentration, a stock solution of dipicolinic acid was prepared in concentrated hydrochloric acid and 1.00 ml samples were placed in 10-ml volumetric flasks. A measured quantity of distilled water was added to each flask, and a duplicate amount of water was added to an empty 10-ml volumetric flask. The flasks were then made up to volume with concentrated hydrochloric acid. The solution in the duplicate flask contained an equal quantity of water, but no dipicolinic acid was used as the reference.

The ultraviolet absorption spectra of dipicolinic acid was also obtained using distilled water and 95 percent ethanol as the solvent. These spectra are also shown in figure 17.

Reaction of 3-hydroxybutanoic Acid in Concentrated Hydrochloric Acid

In experiments with 3-hydroxybutanoic acid, using concentrated sulfuric acid at 100°C, the following results were obtained: Immediately after dissolving commercial 3-hydroxybutanoic acid in concentrated hydrochloric acid, the compound exhibited a single absorption maximum at 256 mμ with a probable molar absorbance index of 5×10^2. Within five minutes after dissolving and with the solution still at room temperature, two absorption maxima were present. These were at 256 mμ and 127 mμ. The latter absorption increased during the first 60 minutes. Continued heating for more than five hours did not result in complete conversion to the new compound which was assumed to be crotonic acid. Assuming the crotonic acid conversion to be 95 percent complete after five hours, the molar absorbance index was calculated to be about 1.3×10^3.

It is quite apparent from these results that absorbance of the hydroxy acid is too low to provide a sensitive spectrophotometric analysis method. Conversion to crotonic acid increases somewhat detection sensitivity. The conversion to crotonic acid is not of sufficient rapidity in concentrated hydrochloric acid to take advantage of the higher sensitivity afforded by its intense peak. At present, it would appear that any micro analysis for the hydroxy acid should be based upon the separation of the polymer and the subsequent reaction of the polymer with concentrated sulfuric acid.

Reaction of Hexoses and Pentoses in Concentrated Hydrochloric Acid

Several sugars were dehydrated in an acid solution containing 15 percent hydrochloric and 10 percent sulfuric acids by volume. The dehydration was carried out in a boiling water bath at 100°C. By extracting samples at 5- or 10- minute intervals it was found that: (a) ribose is converted to furfural in 60 minutes ± three minutes. The furfural shows absorbance maxima in this solution at 283 mμ and 232 mμ with a molar absorbance index of 9.3×10^3;

(b) the reaction is complete in ten minutes ± three minutes and is followed by rapid conversion to levulinic acid. Levulinic acid only is present after 60 minutes. The spectra of the 5-(hydroxymethyl) furfural is similar to furfural with absorption maxima occurring at 235 mμ and 286 mμ; (c) the conversion of both galactose and glucose to 5-(hydroxymethyl) furfural and the further oxidation to levulinic acid appears in both cases to proceed at very nearly the same rate; (d) and more than three hours are required for the reaction to be complete. No significant amounts of the furfural derivative were detected spectrophotometrically at any time during the dehydration of glucose or galactose.

Gas Chromatographic Study of Protein Amino Acids

In spite of the failure of the boron trifluoride esterification treatment of the amino acid, diaminopimelic acid, an exploratory exeriment was performed to see whethor the formation of methyl esters could be observed with a protein hydrolyzate. Wheat gliadin was obtained from California Biochemical Research Laboratories. This is a purified protein made up of 13 amino acids.

A small portion of the gliadin was sealed in a glass ampule with concentrated hydrochloric acid and heated at 200°C for ten minutes. The resulting sample was placed in methanol and treated with boron trichloride, as in the previous boron trichloride work. The resulting chromatogram showed at least 13 peaks. It may be concluded from this experiment that at least a partial methylation of some of the amino acids was obtained with boron trichloride.

B-Hydroxybutyric Acid Oxidation to Acetone

B-hydroxybutyric acid can be oxidized with potassium bichromate and sulfuric acid to acetone. To be able to use this reaction in connection with a gas chromatographic technique, an attempt was made to react the acid with solid CrO_2 supported on inert material such as alumina. The polymer of the acid is soluble in chloroform, glacial acetic acid, pyridine, and octyl alcohol. Glacial acetic acid was chosen as the solvent for this system. In a small distillation apparatus, 15 g of activated alumina (20/30 mesh) was added to the flask, and a 10 percent solution of CrO_2 in glacial acetic acid was then added. Then, the flask was connected to a cold finger and heated with an oil bath until all the acetic acid distilled off, leaving dried alumina which was colored orange. Next, the thermometer was lifted and, with a syringe, 2 ml of glacial acetic acid containing 0.1 percent B-hydroxybutyric acid was injected in the flask and the thermometer returned. The distilled acetic acid was drawn off with the side tube and stopcock.

To detect the acetone produced, six drops of the acetic acid solution was diluted with 20 ml of distilled water, and 2 ml of 40 percent NaOH solution were added. Then, two drops of 10 percent salicyl aldehyde solution in ethanol were added and mixed. If acetone is present, dihydroxydibenzol acetone is formed and this compound has a red color. A blank was prepared in the same manner using six drops of the acetic acid, which was drawn off after the addition of the CrO_2 solution to the alumina. The sample gave a dark orange-red color compared to an orange color of the blank. This showed that the acid can be transformed to acetone in a solid reactor. The advantage of this feature over the competitive solution conversion in potassium bichromate and sulfuric acid is apparent when such a method must be designed for field use.

To study if the reaction is fast, the same test was repeated, but the first three drops of acetic acid were used for the acetone test; this test was also positive for acetone.

3.4 Pyrolysis Studies

A 25 mg sample of Serratia marcescens was placed in a fused silica tube, sealed at one end, and covered with silicone carbide. A stopcock was connected to the other end. The tube was evacuated and hydrogen gas was admitted to a pressure of approximately 200 mm Hg. The end of the tube containing the bacteria and silicon carbide was heated in a gas-oxygen flame to white heat (about 1200°C). Some waxy material condensed on the upper portions of the tube. The room-temperature, volatile-pyrolysis products were then analyzed by mass spectrometry and gas chromatography.

Figure 18 shows a typical mass spectrum obtained at an ionizing voltage of 15.5 ev. This ionizing voltage essentially eliminates oxygen, nitrogen, and hydrogen from the mass spectrum and simplifies the spectrum[33] so that the components can be identified more readily. From this spectrum, it was concluded that the following components were present: carbon monoxide, methane, ethane, ammonia, water, butane, and carbon dioxide. Some higher-molecular-weight, unidentifiable materials were also present.

A gas-solid chromatographic analysis was also performed on these products. This chromatogram is shown in figure 19. A Linde 13X molecular sieve column was temperature programmed as shown in this illustration. Identification of the eluted components was positive and showed the components to be (in order of abundance): carbon monoxide, carbon dioxide, methane, ethane, and butane. Water and ammonia are not eluted as distinct peaks from molecular sieve columns, nor are higher-molecular-weight components eluted at these temperatures.

3.5 Atmospheric Background

Electrostatic precipitation of airborne particles was accomplished using a Micronair Electrostatic Precipitator (Precipitator Corporation of America) and a similar unit built at Melpar (figure 20). This unit has an airflow of 3 million liters per day and appears to collect at 100 percent efficiency. Several suburban locations near Washington, D.C., were sampled. Sampling was conducted at various times from the latter part of May through August. No attempt was made to correlate the concentration of airborne particles with corresponding weather conditions.

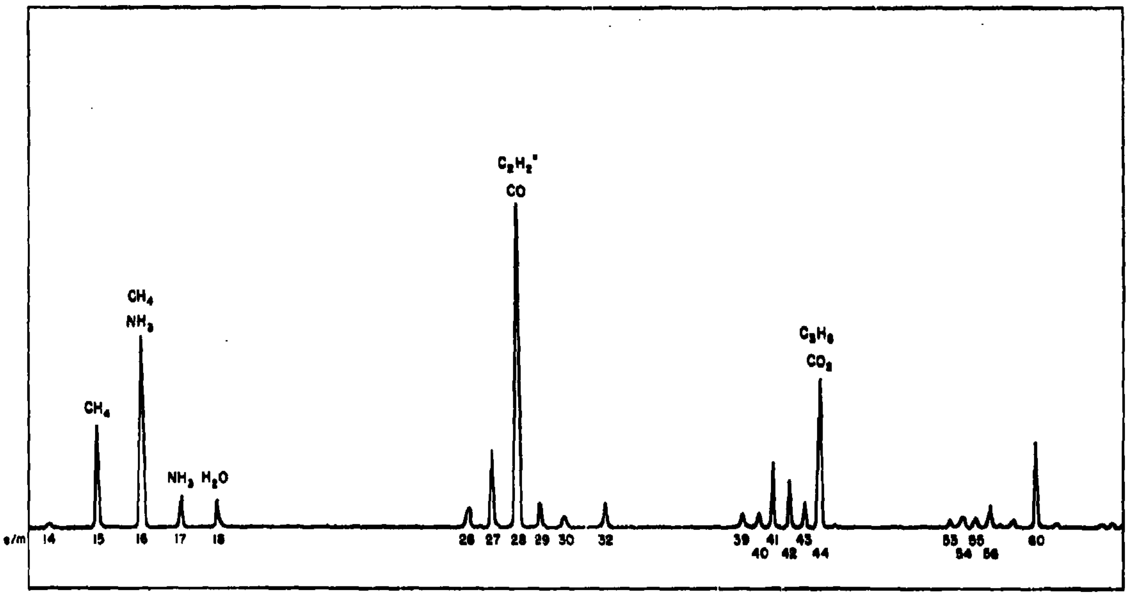

Figure 18. Mass Spectrum of Pyrolysis Products from Serratia marcescens

Conditions: pyrolysis, 1200°C under H_2.
Mass Spectrometry: at ionizing voltage of 15.5 ev.

R4303

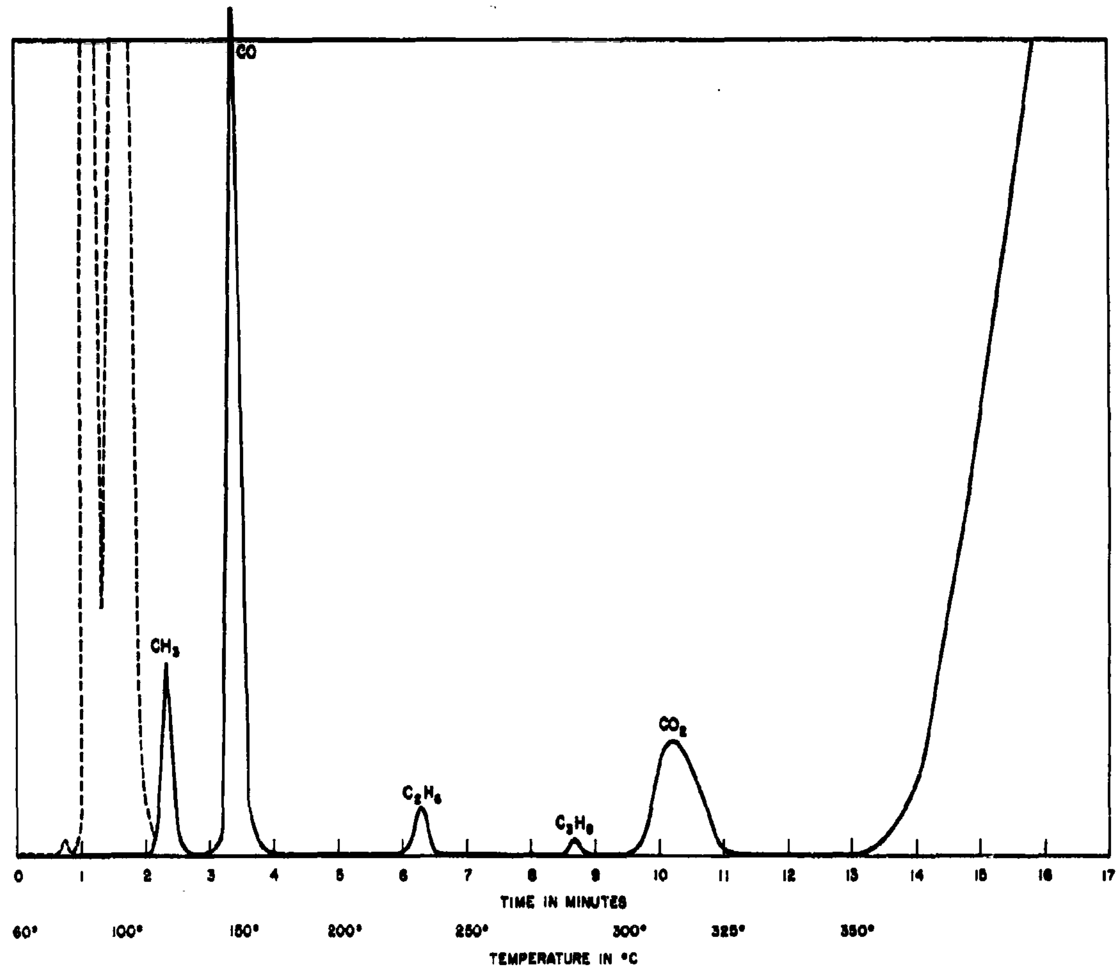

Figure 19. Gas Chromatogram of Pyrolysis Products from Serratia marcescens

Column, 1/8" x 8' molecular sieve 13X, temperature programmed.

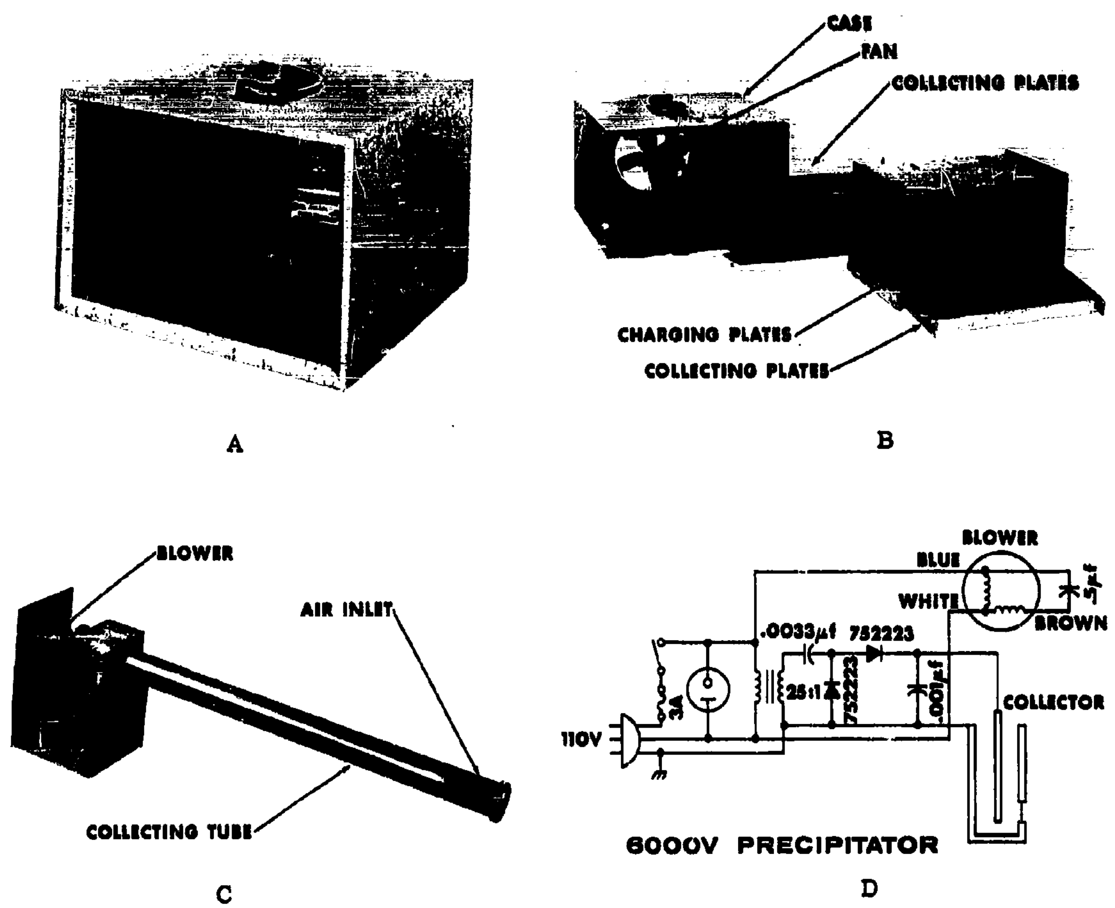

Figure 20. Electrostatic Precipitators

A. Front view of Micronaire Unit
B. Exploded View of Micronaire Unit
C. View of Tube Unit
D. Schematic Wiring diagram of Tube Unit

After collecting for an allotted time, the precipitator was disassembled, cleaned with acetone, and the acetone evaporated at room temperature. The acetone solvent was necessary to achieve complete removal of the collected material from the collecting plates. After evaporation of the acetone, the material was weighed, transferred to the apparatus shown in figure 6 and an extraction-transesterification performed using BCl_3 in the manner described previously for bacteria. Chromatograms were then obtained.

In another test, microscope slides were placed on the collecting plates for the allotted collection time. These were spaced in line with the airflow to determine the efficiency of collection as well as to provide an identification of the types of particles collected. The collected material was fixed to the slide with gentle heating and stained with methylene blue.

The results indicated that atmospheric background concentration during the period analyzed averaged 3.2×10^{-8} g/liter. During the periods of collection, the background varied from a low of 2×10^{-8} g/liter to a high of 5.3×10^{-8} g/liter. The equivalent concentration of bacteria in the background samples was estimated by comparison of the C_{10} to C_{20} methyl ester chromatographic responses with those obtained from Serratia narcescens. The average value corresponded to 11 percent bacteria. The estimated values varied from a low of 4 percent to a high of 25 percent. On this basis, the bacterial (or biological) atmospheric background amounts to about 3.5×10^{-9} g/liter, which is about 1000 to 10,000 bacteria. In all atmospheric background chromatograms, a chromatographic response corresponding to a C_{22} fatty acid methyl ester was noted. This component was presented in amounts which varied from a level about equal to the concentration of the remaining C_{10} to C_{20} esters to amounts present at more than 100 times that of all the C_{10} to C_{20} esters combined. We have made no effort to identify this component as yet. Typical chromatograms are shown in figure 21; table I shows a set of values obtained for the background concentration during August.

Microscopic examination of the collected and stained background material indicated that more than 90 percent of the particles were smaller than five microns. No reliable estimate could be made as to the percent of the total weight represented by the particles smaller than five microns. Figure 22 shows typical photomicrographs of the collected material.

3.6 Gas Chromatography

3.6.1 Columns

Several types of partition columns were used during these studies. These included two nonpolar liquid phases: silicone rubber (General Electric SE-30) and the ether soluble portion of Apiezon W, and a polar liquid phase, diethylene glycol succinate. The nonpolar phases separated essentially according to boiling points, while the polar phases separated both according to boiling

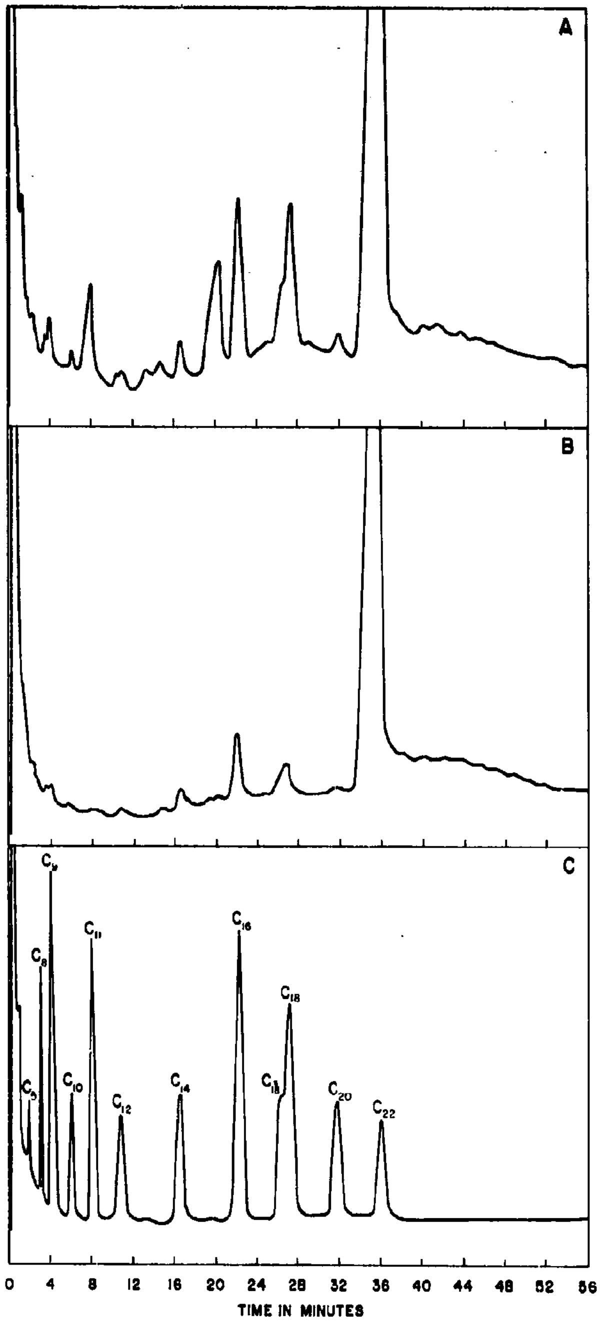

Figure 21. Typical Chromatograms of Methyl Esters from Atmospheric Background

A and B are background while C is comparison against standard mixtures of methyl esters.
Column conditions same as Figure 8.

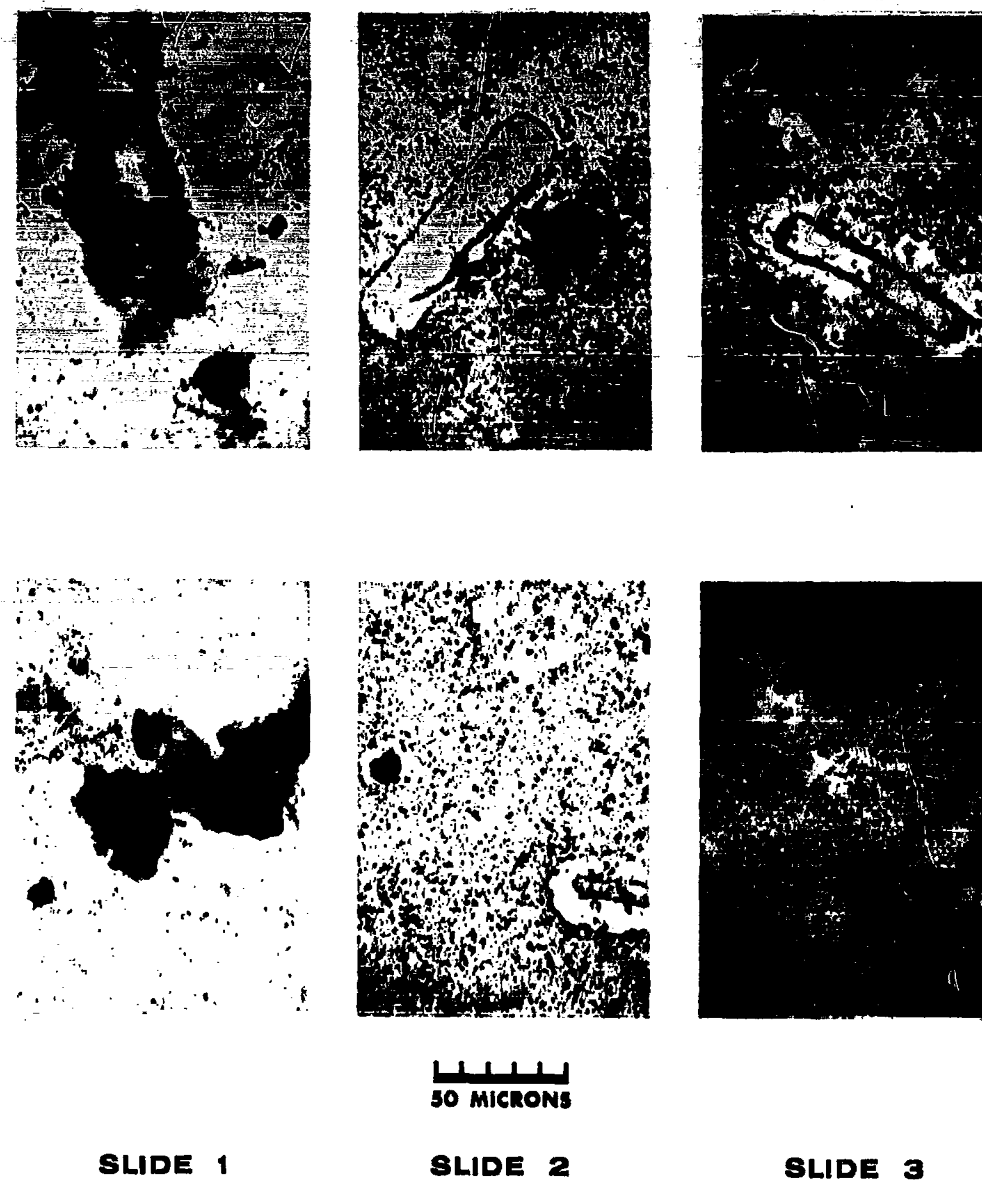

Figure 22. Photomicrographs of Electrostatically Precipitated Atmospheric Background

Stained with methylene blue. Slide 1 near input, slide 2 and slide 3 progressively further from input.

points and degree of unsaturation. The silicone rubber can be used to 400°C maximum, while the diethylene glycol succinate is limited to about 250°C maximum. The Apiezon W could be operated at about 300°. The proportion of liquid phase to solid support used was varied from 1:100 to 10:100 by weight.

Several types of diatomaceous earth supports were used. These included Chromosorb types P and W (Johns-Manville), Gas Chrom type P (Applied Science Laboratories), and Anakrom ABS (Analytical Engineering Laboratories). Anakrom ABC is an acid and base washed material which has been treated with a silicone product to cover polar adsorption sites. It has been the most satisfactory support. The support is coated by dissolving the liquid phase in a suitable solvent, adding the solid support, and stirring with slight heating until the solvent has evaporated. It is then heated overnight at 120°, after which it is carefully sieved to remove lumps and fines. The columns are then packed by applying suction at one end by means of a mechanical vacuum pump while adding packing from the other end. A small hand-held vibrator is used to ensure tight packing while adding the coated support to the tube.

3.6.2 Sample Introduction

In all cases, the lipids, fatty acids, or esters were dissolved in ether and injected through a silicone rubber septum into a flash evaporator maintained above the boiling points of the components.

Table I

ATMOSPHERIC SAMPLES COLLECTED BY ELECTROSTATIC PRECIPITATION

Sample	Concentraction in g/liter	Percent bacteria (estimated)
1	2×10^{-8}	12
2	4×10^{-8}	4
3	2.2×10^{-8}	10
4	4.9×10^{-8}	25
5	5.3×10^{-8}	12
6	2.1×10^{-8}	17
7	2.6×10^{-8}	17
8	2.4×10^{-8}	10

3.6.3 High-Resolution, High-Speed Chromatography

It was noted that a short column, when temperature-programmed from 125° to 300°, provided resolution that was comparable to that obtained with much longer columns of the same diameter similarly programmed. On this basis, it was reasoned that, by decreasing the diameter of the column, a more even heating of the column packing from the exterior of the column to the center would occur; consequently, there would be a more even change of partition coefficients. In other words, a smaller-diameter column should be more conducive to faster-temperature programming. This concept was tested using a Beckman Thermotrac linear-nonlinear temperature programming oven in conjunction with a Beckman hydrogen-flame detector. A 22 inch x 1/16 inch ID brass column was packed with 160/170 mesh Anakrom ABS initially coated with 2% SE-30 silicone rubber. After conditioning, typical chromatograms as seen in figure 23 were obtained when either solutions of the fatty acid methyl esters from dairy butter were injected into the column and the column temperature programmed rapidly as indicated by the dotted lines in the chromatograms. The small-diameter column allows much faster programming. Thus, the optimum programming rate for a 1/4-inch-diameter column was found to be 5°/minute, while for a 1/16-inch-diameter column the optimum programming rate was about 20°/minute.

These chromatograms were obtained by injecting small ether volumes (1 to 5 µl) containing a relatively high concentration of methyl esters with the detector operating at an extremely insensitive attenuation (signal attenuated 50 x 10^4 times). When larger volumes of ether containing smaller concentrations of methyl esters were injected onto the column, additional problems arise if the hydrogen flame detector is operated at high sensitivities. These problems are discussed below.

3.6.4 Column Stability

Although silicone rubber is considered to be thermally stable to 375° when used as a liquid phase in gas chromatography, column bleeding is evident at temperatures above 300°. At such temperatures, the volatile materials are probably compounds which are strongly retained by the silicone rubber and, as a result, bleed off at a more or less constant rate as they are carried very slowly through the column. At higher temperatures, the silicone rubber undoubtedly is continually being decomposed slightly to produce smaller molecules of sufficiently high vapor pressure to pass through the column.

A column can be cleaned of objectionable molecules in the case of normal bleeding by using a gas phase other than helium (or mixed with helium) to shift the degree of partition of strongly retarded compounds from the liquid toward the gas phase. We have found that steam can sometimes be used successfully. In fact, a generator for producing steam as a carrier gas in gas chromatography is commercially available for use with

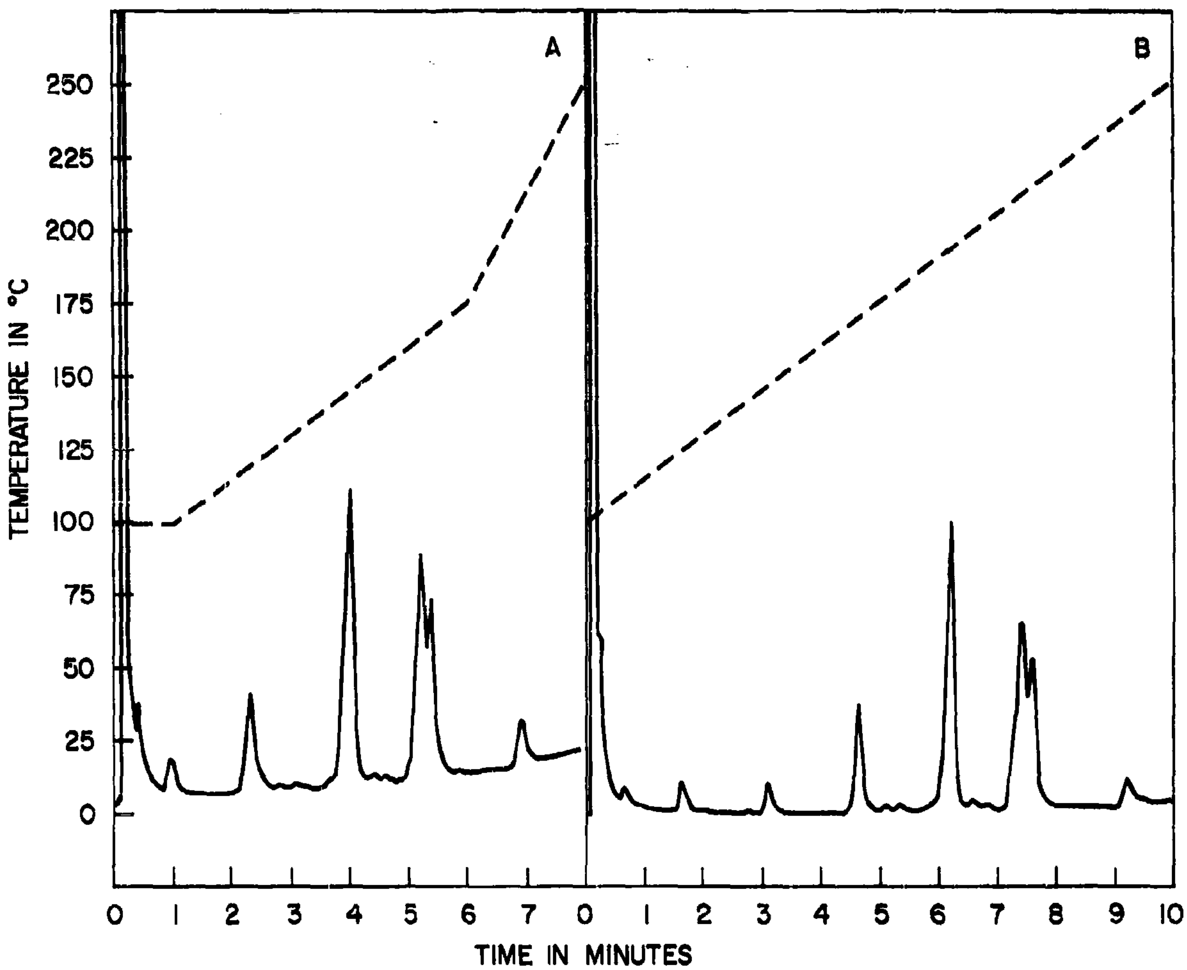

Figure 23. Rapid-Programmed Temperature Chromatograms of Butter Fatty Acid Methyl Esters

Conditions: 22-inch x 1/16-inch ID column initially containing 2% SR on Ankrom ABS, 160/170 mesh. Hydrogen-flame detection. Temperature programmed as shown by dotted lines.

the hydrogen-flame detector (Wilkens Instrument and Research, Inc.). A high-boiling, organic compound which has relatively low solubility in silicone rubber can also be used if the column temperatures are maintained above the boiling point of that liquid. We have successfully used p-bromophenetole in this connection. In both cases, the column is heated to the desired temperature and, with helium flowing through the column, large samples (several ml) of water or the organic liquid are injected into the hot injection port.

Ether and other organic solvents, in which silicone rubber is soluble, appear to lead to a partial removal of the silicone rubber as well as the impurities. At high hydrogen flame-detector sensitivities, even very small ether injections (1 to 10 μl) result in undesirable background because of increased column bleeding; this bleeding was manifested as extreme trailing and a baseline shift upscale. Larger ether solutions of the fatty acid methyl esters from bacteria show this effect with the less sensitive thermal conductivity detector. An example is shown in figure 24. It will be noted that trailing in the chromatogram continues for more than ten minutes. It has been concluded that injection of the fatty acid methyl esters in ether solutions is not feasible when high-detection sensitivity is required.

One of the most time-consuming portions of the extraction-transesterification-gas chromatography of bacterial fatty acid methyl esters is the removal of BCl_3 and BCl_3-methanol reaction products from the transesterification reaction mixture. It has been postulated that these reaction products include methoxy boron compounds and alkyl chloride structures which are formed with the release of hydrogen chloride. We have used water to hydrolyze these reaction products. The esters are extracted from the treated solution with ether.

The BCl_3 and BCl_3-methanol compounds are volatile at low temperatures. The boric acid formed in the dehydration reaction is not volatile. The direct injection of the reaction mixture is an appealing method of shortening the total analysis time. Unfortunately, pure methanol leads to column bleeding in a manner similar to ether, while BCl_3 seems to catalyze the decomposition of the silicone rubber.

The commercial hydrogen-flame detector used in these studies has a zero suppression control which can be used to cancel out the background caused by column bleeding. However, as the column is temperature-programmed from, for example, 50° to 350°C, the column bleed rate will change from zero to some extremely large value; this change in bleed rate is easily seen by the detector. Accordingly, constant zero suppression cannot be used with a temperature-programmed column at high-detector sensitivity. It would seem at first that programming the zero suppression to follow the column-temperature program might be feasible. Unfortunately, variations in the size of sample injected and the fact that background

R3951

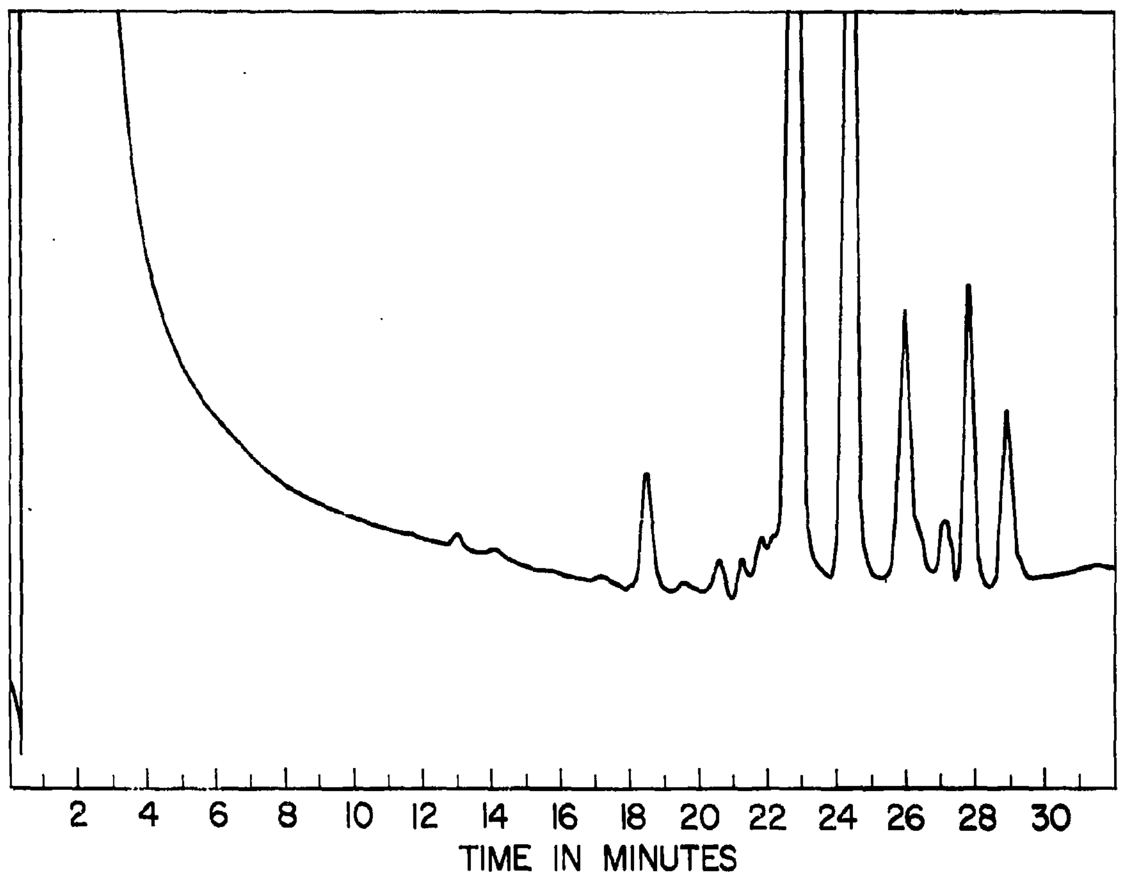

Figure 24. Effect of Addition of Large Ether Samples on Gas Chromatograms

300 μl ether containing fatty acid methyl esters from 50 mg S. marcescens. Column is 5% SR on Anakrom ABS, Conditions same as Figure 12.

electrical noise increases with increasing zero suppression would preclude this programming. At the higher temperatures, the background noise may easily be greater than the signal response of the recorder when the zero suppression is used.

We have found that under normal operating conditions, when ether injections larger than about 10 to 20 µl are eluted from the column, the hydrogen flame is almost invariably extinguished.

From these studies it has been concluded that, to achieve the sensitivity of which the hydrogen flame detector is inherently capable, the following conditions must be met: (a) a sampling system which does not require the passage of a solvent through the column must be used; (b) columns containing thin liquid coatings on the solid support for short retention times and low zero suppression is required at the higher temperatures; (c) for rapid temperature programming, the distance from the outside of the column to the center of the column must be kept as small as possible (capillary tubing would seem to be ideal); (d) if zero suppression is required, the column must be operated isothermally to avoid extreme baseline drift.

A system incorporating most of the features indicated above has been designed, constructed, and the principle tested. It is illustrated and discussed briefly in figure 25.

The results showed that it is possible to inject a solution of C_5 methyl ester in ether and vent off the ether without losing any appreciable amount of the C_5 ester (see figure 26). The C_5 methyl ester (methyl pentanoate) has a boiling point of 127° at atmospheric pressure, and ether (diethyl ether) has a boiling point of 35°. Methanol, the methyl borates, and boron trichloride all have boiling points below 79° and should present no problems. A direct injection of the BCl_3-methanol-bacteria reaction mixture, however, was not attempted.

The precolumn (ester trapping column) cannot contain a packing which will adsorb any of the molecules. This is illustrated in figure 27, which shows the effect of using alumina instead of silicon carbide to provide a surface for the condensation of the esters. It will be noted that not only is ether retained, but in addition, the methyl esters are not resolved.

At higher detection sensitivities than that used in figures 26 and 27, opening and closing the vent valve seriously disrupted the chromatogram. This is probably caused by heating and cooling of the hydrogen flame as the carrier gas flow was turned off and back on. With the 1/8-inch OD packed column used in these studies, the flow rate is appreciable to 60 ml/minute). It was believed that capillary columns might avoid this effect because the flow through a capillary column may be 10 to 100 times less.

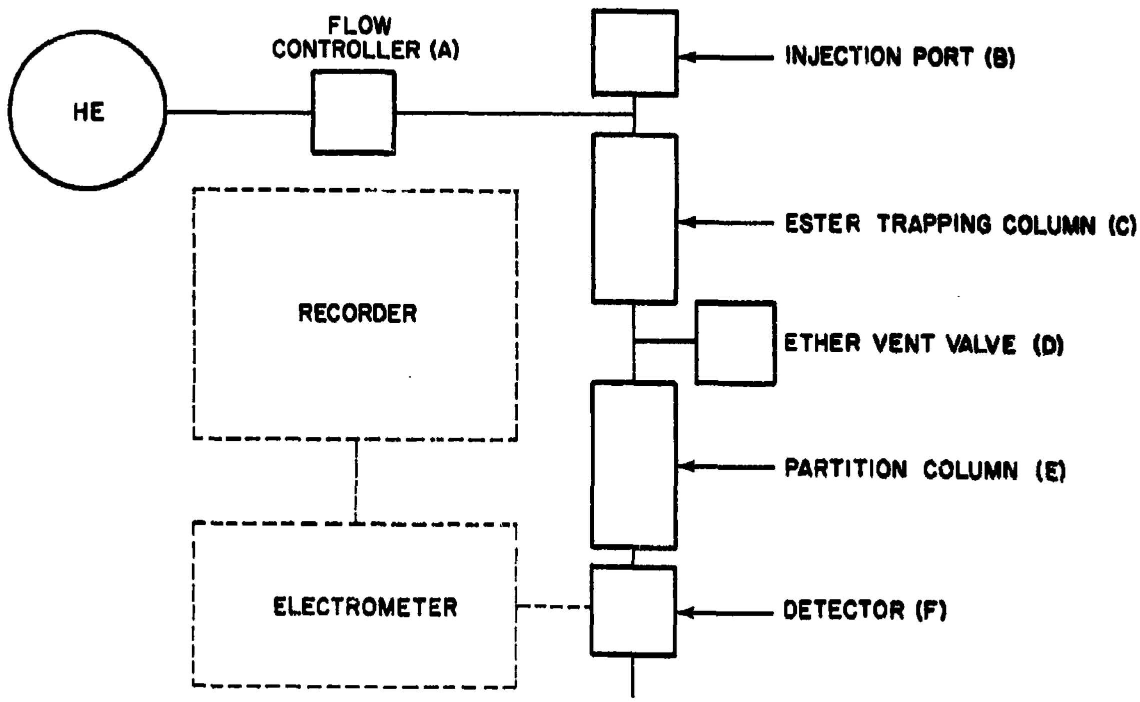

Figure 25. Proposed Chromatograph for Achieving Increased Sensitivity and Decreased Analysis Time

An ether or methanol-BCl_3 solution of methyl esters is injected at B with C at room temperature and D vented to the atmosphere. After flushing out the solvent through D, D is closed directing the gas flow through E and F. C is then heated rapidly to about 300°C resulting in transfer of the esters to E. The esters are eluted from E and sensed with detector F.

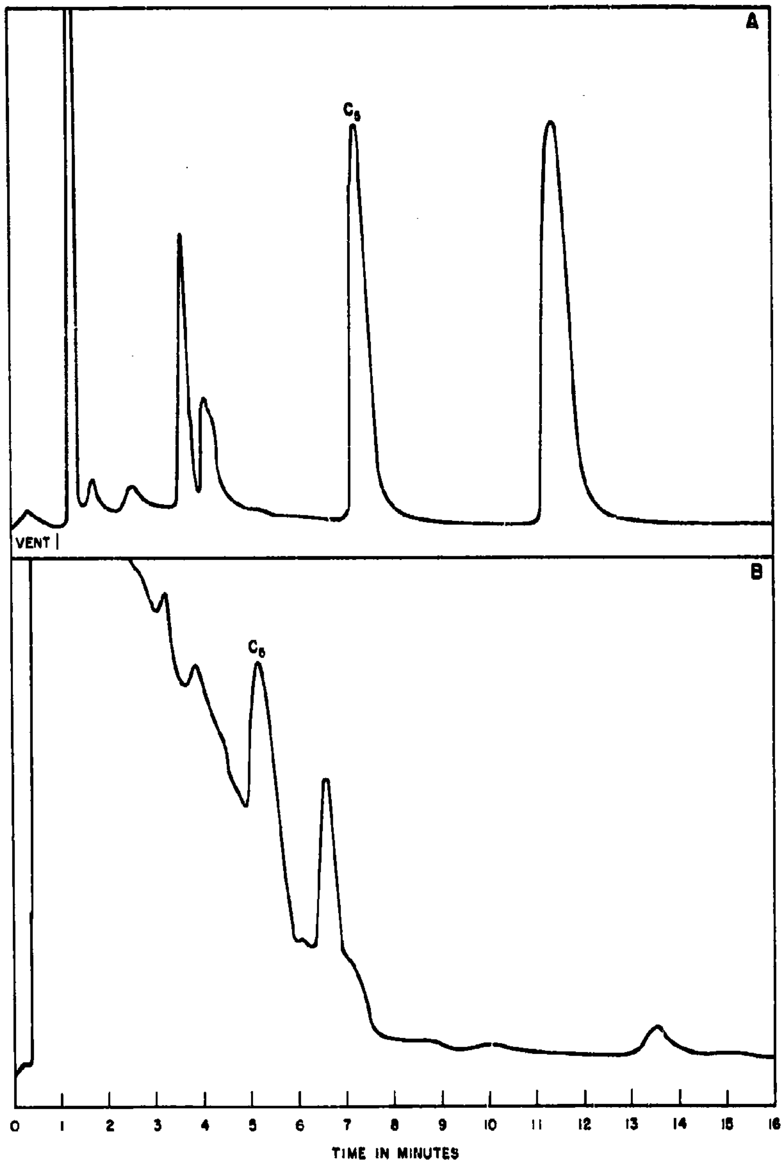

Figure 26. Methyl Ester Chromatogram With and Without Solvent Venting

Conditions: Column 1/8'' x 1.5 meter, 5% SR on 160/170 Anakrom ABS, isothermal at 70°C. Sample size 30μl containing 2.5 x 10^{-7} g/μl. of C_5 methyl ester. Hydrogen flame detector, electrometer 1/5000 maximum sensitivity. Precolumn filled with silicon carbide 100/120 mesh.

A. with venting

B. without venting

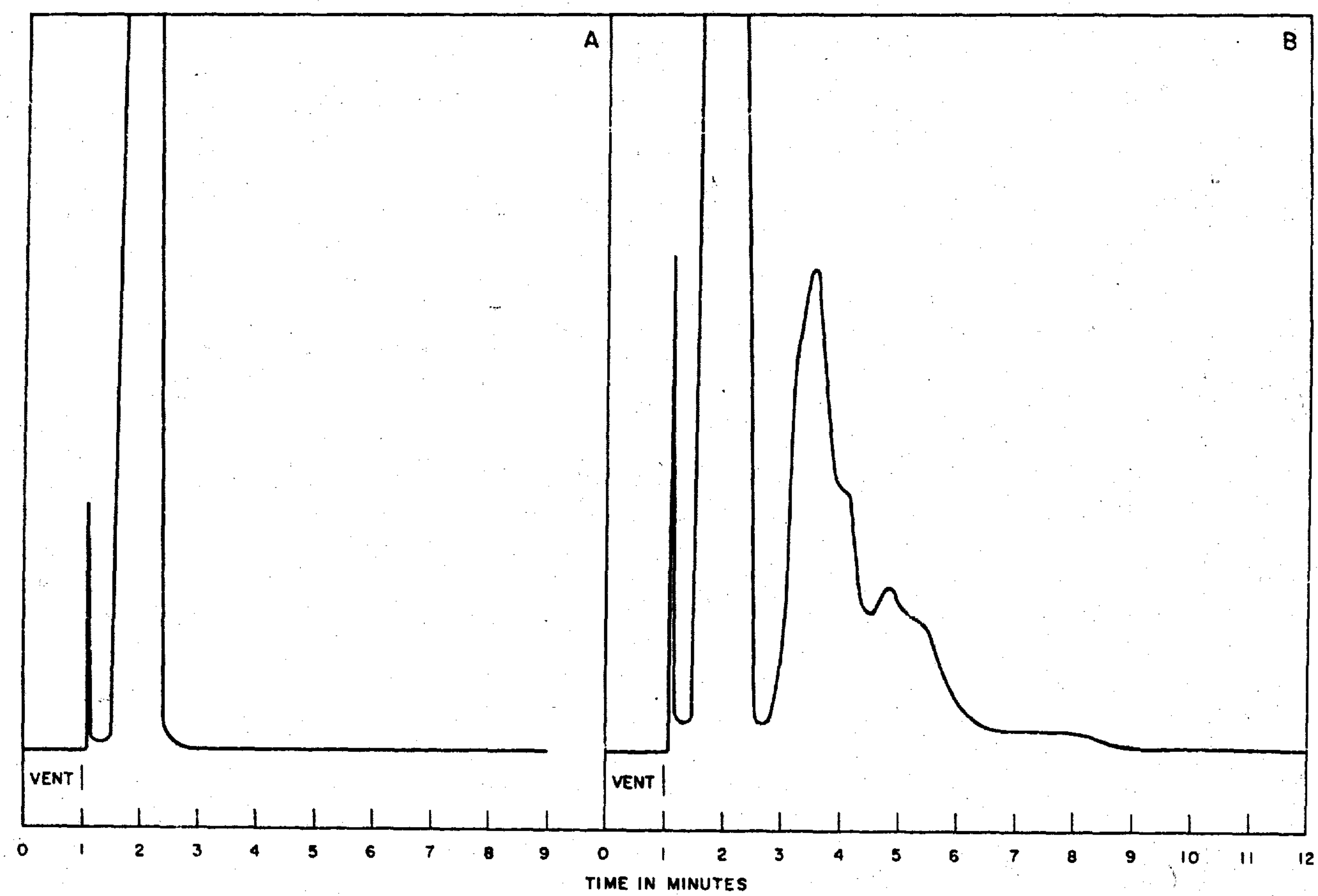

Figure 27. Effect of Alumina in Precolumn

Conditions: Same as figure 25 except precolumn was filled with 100/120 mesh alumina.

A. 30μl ether with venting

B. 30μl of other solution of C_5 methyl ester with venting

Figure 28 shows the behavior of a mixture of methyl esters using the sampling technique above.

An experimental gas chromatograph was designed and constructed to provide for easy adaptation to a variety of experimental injection systems, columns, and detectors. Photographs of this design are shown in figure 29, along with the schematic electrical diagram. A precolumn consisting of 4 feet of 1/16-inch OD by 0.020-inch ID capillary tubing was used for this application. It was electrically insulated at one end and connected to a 6-volt AC source. Under these conditions, the column itself became a resistive heating element. In this way, extremely rapid heating of the precolumn was achieved. Because of the detector and, to some extent, electrometer troubles, it was impossible to check this instrument at this time for sensitivity and speed.

3.6.5 Argon Ionization Detector

According to Lovelock,[34] the argon ionisation detector should be approximately 10^3 times as sensitive as the hydrogen-flame detector. Its performance is seriously impaired by the presence of air or water vapor in the carrier gas and, as a consequence, this higher sensitivity is seldom realized. It also has a smaller range over which its response is linear with sample concentration. The design and construction of the detector also affect its sensitivity.

Tritium embedded in a strip of metal is generally used as the beta emitter for the argon ionization detector. The detector temperature, however, must be maintained above 200° for the methyl ester analysis to avoid condensation within the detector. At these temperatures, tritium is rapidly removed from the metal strip. Accordingly, a different emitter must be used. Sources that can be used at this source at 200°C and higher include SR^{90}, Pm^{147}, Ra^{226}, and Ra-D.

For a preliminary feasibility determination, a Barber-Coleman argon ionization detector, utilizing Ra^{226} as the ionizing source, was used. This detector was found to be unsuitable. At an electrometer sensitivity which provided a noise level of 5 percent on a 1 mv recorder, the maximum detection sensitivity was only 10^{-6} grams for a C_{14} methyl ester. This would correspond to only about 10^{-6} gram of bacteria. In addition, increasing the sample concentration by only a factor of 10 to 100 times the maximum detection sensitivity at this moise level resulted in anomolous behavior as shown in figure 30. In the chromatograms of this figure, peak reversal is seen. This reversal has been accounted for by assuming that, up to a certain concentration, the molecules begin to absorb or capture the electrons and a reverse peak occurs. Accordingly, these should be a region at which the compound ionization and electron capture aspects are equal and no response will occur. An approach to this situation can be seen in figure 30.

R4306

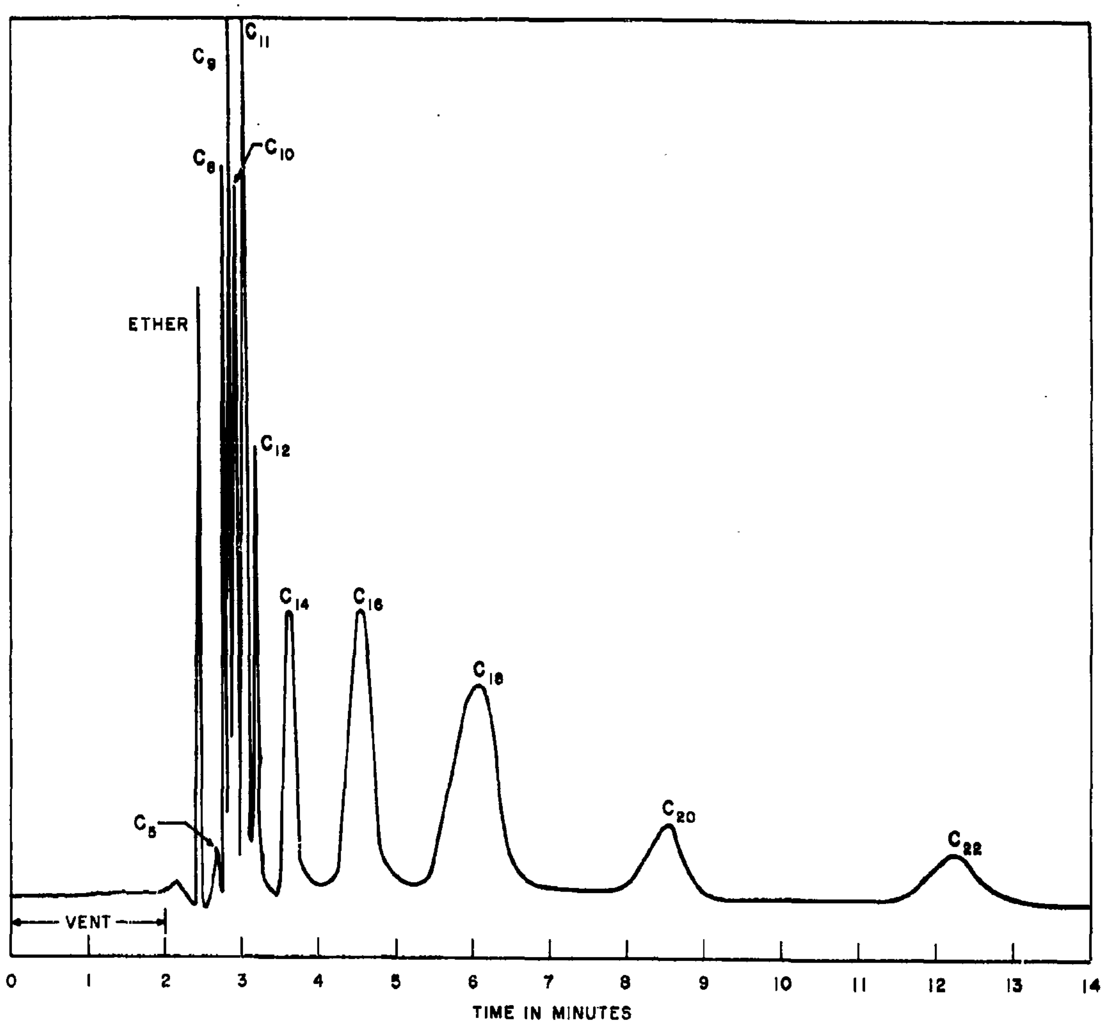

Figure 28. Chromatogram of Methyl Ester Mixture

Same conditions as figure 25 except column temperature 180°C.

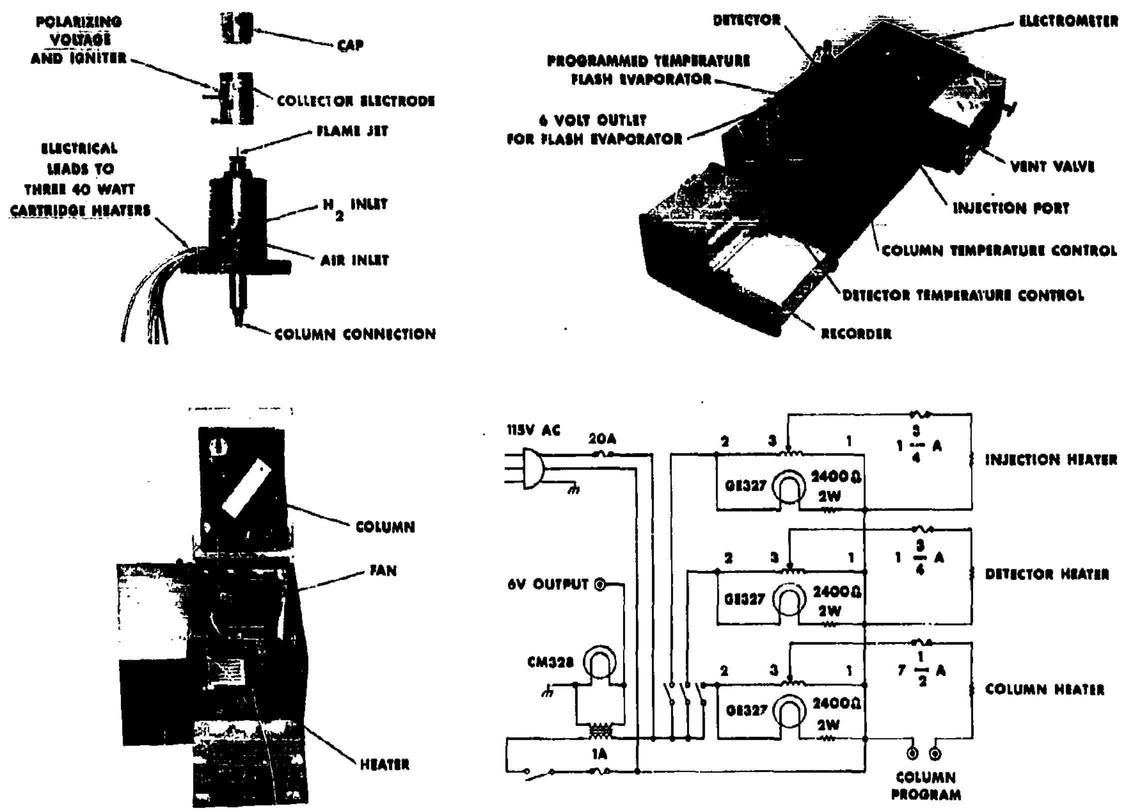

Figure 29. Experimental Gas Chromatograph

A. Hydrogen Flame detector, exploded view.
B. Assembled Gas Chromatograph
C. Column Oven Chamber
D. Schematic Wiring diagram of Column Oven

R4307

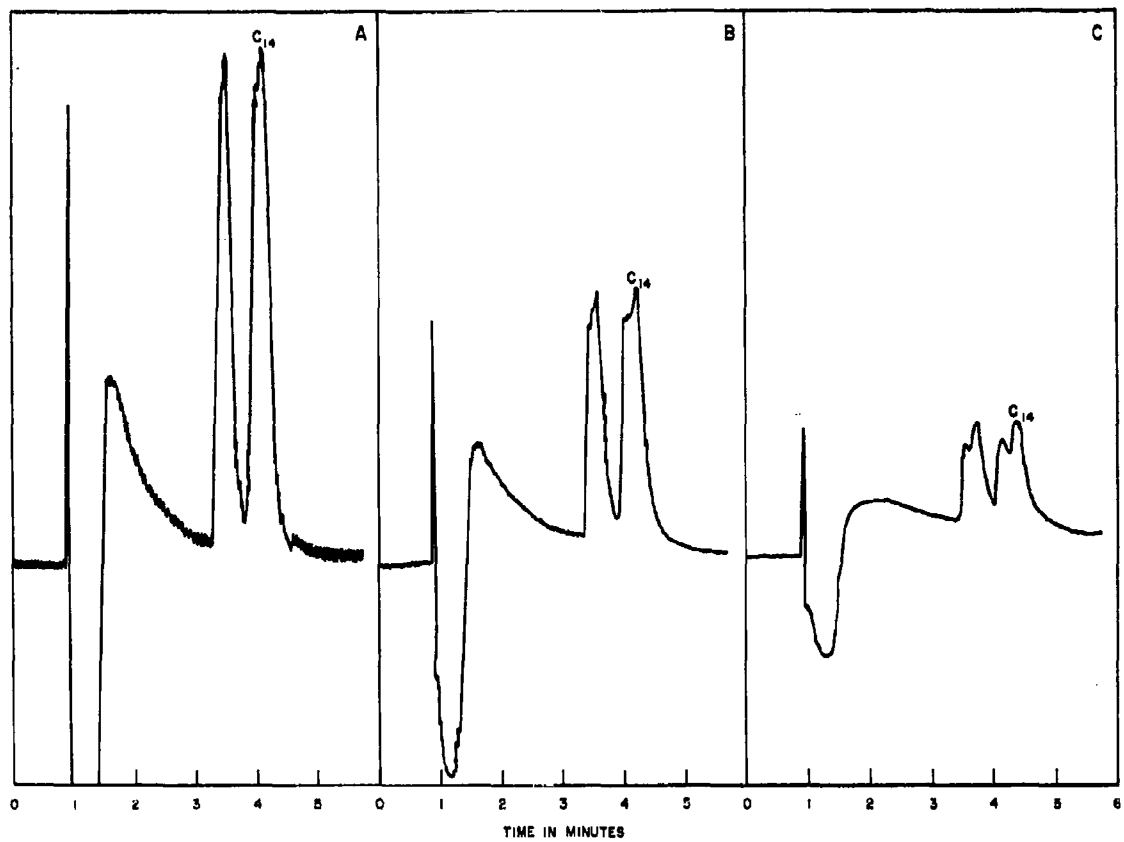

Figure 30. Effect of Argon Ionization Detector Overloading

Column is SE-30 polyester. Isothermal at 225°C.
A. 1×10^{-5}g. of C_{14} in 0.4μl ether, 1/1600 maximum electrometer sensitivity.
B. 2×10^{-5}g. of C_{14} in 0.8μl ether, 1/800 maximum electrometer sensitivity.
C. 4×10^{-5}g. of C_{14} in 1/6μl ether, 1/400 maximum electrometer sensitivity.

It is possible that the high noise level is associated with the emission of alpha and gamma rays from the radium. The detector design may also be involved. No attempt has been made to modify the detector.

3.6.6 H_2- Flame Detector Sensitivity

A preliminary study was made of the practical sensitivity of the Beckman hydrogen-flame detector and electrometer. For this, a typical C_{14} methyl ester was used with the electrometer attentuated to 1/5000 the maximum sensitivity. By extrapolating the results to maximum sensitivity, a value of 3.6×10^{-11} gram of C_{14} fatty acid should be realized assuming a signal-to-noise ratio of 10 to 1 for detection. This would correspond to approximately 5×10^{-9} grams of Serratia marcescens which could be detected. Unfortunately, the noise level of the Beckman unit is very high at maximum sensitivities, as noted in figure 31. In this figure, it can be seen that, for a noise level of 2.5 percent of full-scale response on a 1 mv recorder, an attenuation of 1/50 maximum sensitivity is required. Accordingly, the practical maximum sensitivity of this unit for recording concentration profiles lies between 10^{-8} and 10^{-9} grams of a pure culture of bacteria. Better electronic components and shielding might increase this sensitivity by one or two orders of magnitude.

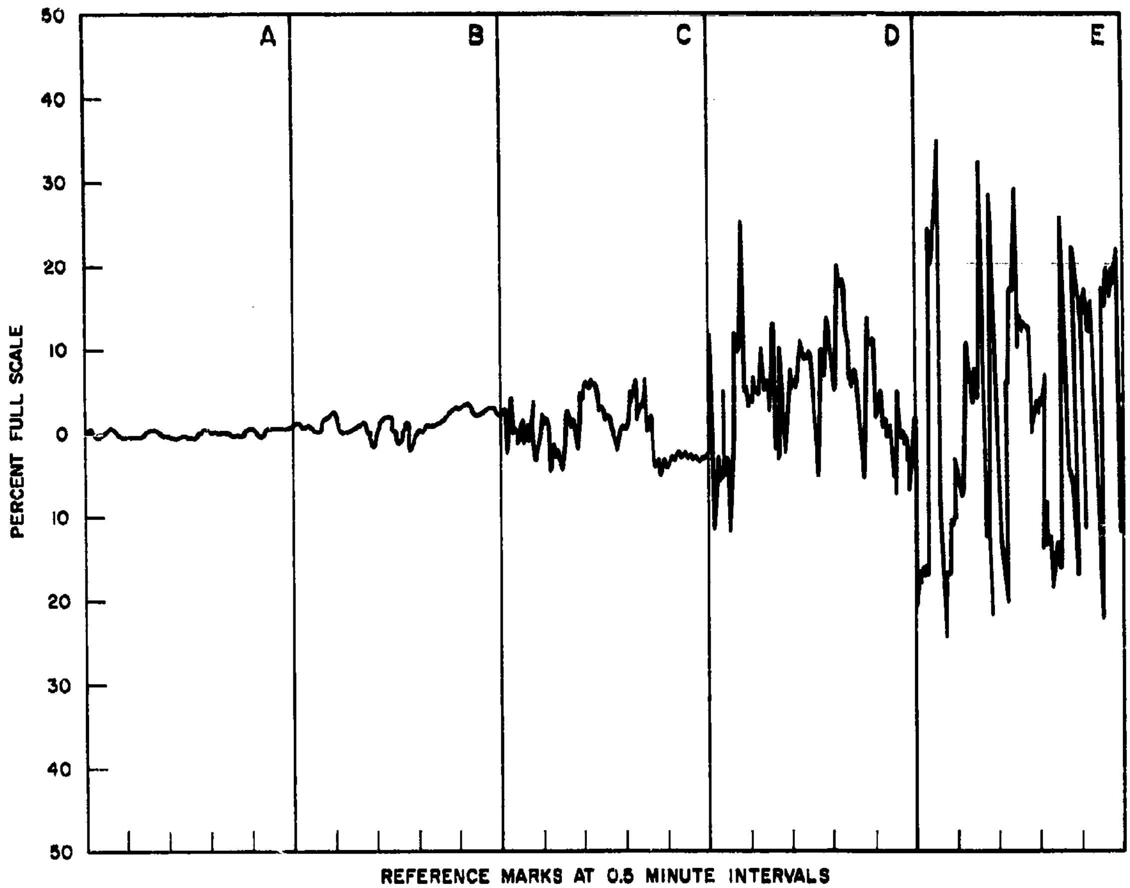

Figure 31. Noise Level for Beckman Hydrogen Flame Detector and Electrometer

1.0 mv full scale response. Column is SE-30 silicone rubber on Anakrom ABS operating at 180°C.
A. 1/50 maximum sensitivity
B. 1/20 maximum sensitivity
C. 1/10 maximum sensitivity
D. 1/5 maximum sensitivity
E. 1/2 maximum sensitivity

4. CONCLUSIONS

Approximately 80 percent of the effort in this feasibility study was directed toward the use of bacterial lipid methyl esters in establishing the concept of the Concentration Profile for the detection and identification of microorganisms. The remainder of the effort was directed toward a brief study of the biological background normally present in the atmosphere and other gas chromatographic, spectrophotometric, pyrolytic, and mass spectrometric techniques as possible methods for use in the Concentration Profile concept.

It has become apparent that the gas chromatographic approach developed during this study has potential in both detection and identification. It has been shown that bacterial families have distinctly similar profiles and that large differences exist among different families. The method shows promise of being very sensitive to pure cultures of bacteria or to cultures which have other bacterial or biological contaminants present in very low concentrations.

The feasibility of the Concentration Profile concept has been demonstrated. The lipid methyl ester Concentration Profile method appears to be potentially capable of use for rapid detection of bacteria. At present, however, it would seem to be more applicable to the identification of bacteria if some additional method of separating mixtures of bacteria into pure cultures were used.

A high background level was noted during the final months of this study. The total particulate concentration in the atmospheric background varied from 2×10^{-8} g/l to 5.3×10^{-8} g/l, while the percentage of particulate material estimated to be bacterial in nature varied from 4 to 25 percent. On this basis, the normal bacterial level could be expected to vary from 8×10^{-10} g/liter (about 1000 organisms) to about 1.3×10^{-8} g/liter (about 10,000 organisms). Because the method appears to have a potential sensitivity of 10^{-9} g bacteria, and because biological aerosols produced for military purposes would result in concentrations of 10^{-9} to 10^{-11} grams of organisms per liter it becomes apparent that background presents a serious problem in the application of this method directly to atmospheric collection without prior separation. Viable organisms are normally present in the atmosphere at concentrations of less than 1 organism/liter. Accordingly, a method which could rapidly and continuously separate atmospheric organisms into viable and nonviable portions would do much to make this or a similar method feasible. The only methods now available for this are those which involve culturing the collected particles for more than 12 hours. Logically, a detection method based on viable organisms would be perferred.

Unlike conventional biological techniques, chemical methods will analyze both viable and nonviable organisms indiscriminately. This is both an advantage and a disadvantage. For zero atmospheric background, it would be an advantage because aerosol production and dissemination results in a relatively high death rate for the organisms, particularly for nonspore formers.[35] On the other hand, as noted above, the normal presence of nonviable bacteria in the atmosphere complicates the analysis.

It is to be concluded that before any Concentration Profile technique making use of variations in chemicals common to all bacteria can be used successfully, a significant amount of effort and research must be directed toward the development of effective, rapid methods of separating bacteria according to species.

Two types of detection methods were investigated during this study: (a) optical property sensors and (b) ionization sensing devices. In the first category can be placed the spectrophotometric methods, while in the second can be placed gas chromatography and mass spectrometry which utilize the formation and collection of ions. Of these two types of detection, the ionization type appears to offer the greatest sensitivity using present state-of-the-art components and techniques.

5. RECOMMENDATIONS FOR FURTHER RESEARCH

5.1 Gas Chromatographic Studies

It is apparent that only "the surface has been scratched" by this feasibility study into the use of the concept of the concentration profile. Indeed, the use of lipid methyl esters and gas chromatography in determining such profiles for the identification of bacteria has, for all practical purposes, only begun. This method, which makes use of a class of compounds present in all microorganisms, is only one of a number which could be practical. For example: The analysis of amino acids through their conversion to aldehydes with subsequent analysis by gas chromatography is feasible.[36, 37] Operating a precolumn of diatomaceous earth impregnated with Ninhydrin at 130°, the amino acids can be transformed instantaneously to the corresponding aldehydes, separated by gas chromatography and detected by hydrogen flame ionization. Similar systems can be envisioned for the carbohydrate portions of microorganisms.

The success of any such method as noted above for microorganism detection would be dependent upon the development of rapid methods of hydrolysis, because present methods require several hours to days for completion.

In this regard, however, it should be recalled that the previous methods of analyzing lipids by gas chromatography also required many hours to days to accomplish. A significant increase in speed has been achieved in this area, and related areas should be just as available to improvement.

Another area similar in nature is the analysis of the products of controlled pyrolysis of bacteria. Janak[38] has pointed out that, when a substance is pyrolyzed in the carrier gas of a gas chromatograph, the products are immediately diluted and, under these conditions, pyrolysis does not follow the normal process that occurs on a larger scale. The method appears to be reproducible and, like other gas chromatographic methods, would not be influenced by the presence of inorganic contaminats.

Total pyrolysis may also offer a method for obtaining distinctive concentration profiles. The total pyrolysis products are, however, fewer in number and would, accordingly, not offer as many variables for monitoring purposes as in the extraction methods discussed.

5.2 Ionization Methods

The most sensitive detection of gas chromatographic components is afforded by ionization methods. In addition, the simple combustion of entire microorganisms in a hydrogen flame and detection of the resulting ions might offer a nondiscriminatory background detecting device which might find some application in these studies. For example: Variations in the background level of atmospheric contaminants resulting from the presence of spores and bacteria would occur from day to day and hour to hour depending upon the climatic conditions as well as the sporolation of plants or fungi. These natural variations would be somewhat gradual, whereas an artificially produced aerosol would be expected to pass a sampling station in less than one minute. The response caused by natural variations, would, accordingly, be a gradual change in readout response, while the response caused by artificially produced aerosol might appeas as a superimposed spike.

The combination of controlled or total pyrolysis combined with simplified mass spectrometric methods could be a powerful approach because a concentration profile of predetermined constituents could be made on a continuous and simultaneous basis. As indicated earlier, the proper choice of operating parameters simplifies the interpretation of mass spectrometric results; as a result mass spectrometry, is more feasible for this application.

5.3 Spectrophotomeric Methods

Fluorescence methods and microspectrophotometric methods are some of the most sensitive methods of analysis. Many common organic materials, however, will interfere with the measurements carried out by this means. As with all methods, collecting, handling, and chemically treating the organisms on such an extreme micro scale would present the most obvious obstacles to use of these methods. On the other hand, it is known that certain chemicals are unique to microorganisms,[1] and it appears highly probable that certain chemicals are unique to pathogenic organisms. Studies into this very fundamental area of research could be facilitated greatly by spectrophotomertic techniques. If such compounds are found, the development of sensitive methods of analysis could also be feasible, particularly if the background material presents a major spectral interference.

5.4 Collection and Separation

5.4.1 Collection

Large capacity collection devices are now available and improved versions are under development. Such devices or methods as electrostatic precipitation and impingement techniques are quite practical. These approaches, however, should have incorporated in them a means for delivering sample to the chemical detection instruments on a continuous basis.

5.4.2 Separation

The high biological background present in the atmosphere indicates that the success of any method based upon the concentration profile of chemicals common to all microorganisms will be largely dependent upon the development of complimentary separatory methods and facilities. Separatory methods might include one or more of the following: (a) separation based on particulate size and/or density to eliminate pollen and inorganic material; (b) separation based upon viability, (because the normal background contains less than one viable microorganism per liter, such a process would simplify detection considerably); (c) separation based upon electrophysical properties, such as migration in an electric field because of surface charge differences.

Impingment techniques are fairly well established as methods for separating airborne particles on the basis of size. Separations based upon viability are established in conventional bioclinical analysis and involve the culturing of collected samples. Unfortunately, this requires many hours to several days. Electrophoretic methods of separating bacteria have been applied with some success. Further work in all of these areas is required.

6. REFERENCES

1. H. Wolochow, ASTIA Report No. 211170, January 15, 1959.

2. A. D. Langmuir, Bact. Rev., 25, 173 (1961).

3. Public Health Monograph No. 60, U.S. Dept. of HEW (1961).

4. A. T. James, Methods of Biochemical Analysis, VIII (ed. by D. Glick), p. 17, Interscience Publishers, Inc., New York, 1960.

5. J. Folch, I. Agcoli, M. Lees, and J. A. Meath, J. Biol. Chem., 191, 8 (1951).

6. R. J. Anderson, Chem. Rev., 29, 225 (1941).

7. C. E. Leyes, Encyclopedia of Chemical Technology, (ed. by R. E. Kirk D. F. Othmer), pp. 777-817, Interscience Publishers, Inc., New York, 1950.

8. H. Kurz, Fette u. Seiten, 44, 144 (1937), reported by A. T. James in Methods of Biochemical Analysis, (ed. by D. Glick), v. 8, p. 18, Interscience Publishers, Inc., New York, 1960.

9. H. E. Carter, R. A. Henry, and N. Z. Stanacek, J. Lipid Research, 2, 223 (1961).

10. W. Stoffel and E. H. Ahrens, Jr., J. A. Chem. Soc., 80, 1950 (1958).

11. I. Hornstein, J. A. Alford, L. E. Elliott, and P. F. Crowe, Anal, Chem., 32, 540 (1960).

12. L. D. Metcalfe and A. A. Schmitz, Anal, Chem., 33, 363 (1961).

13. M. L. Vorbeck, L. R. Mattick, F. A. Lee, and C. S. Pederson, Anal. Chem., 33, 1512 (1961).

14. G. G. Exposito and M. H. Swann. Anal. Chem., 34, 1049 (1962).

15. L. Pauling, The Nature of the Chemical Bond, p. 318, Cornell University Press, 3rd ed., 1960.

16. W. Gerrard and M. F. Lappert, Chem. Rev., 58, 1081 (1958).

17. J. J. Perry and J. W. Foster, J. Bact., 72, 295 (1956).

18. J. F. Powell, Biochem. J., 54, 210 (1953).

19. F. W. Janssen, J. Lund, L. E. Anderson, Science, 127, 26 (1958).

20. J. H. Law and R. A. Slepecky, J. Bact., 82, 33 (1961).

21. M. Lemoigne, Bull. Soc. Chem. Biol., 8, 770 (1926).

22. D. H. Williamson and J. F Wilkinson, J. Gen. Microbiol., 19, 198 (1958).

23. I. J. Duncan, Ind. Eng. Chem. Anal. Ed., 15, 162 (1943).

24. R. A. Stillings and B. L. Browning, Ind. Eng. Chem. Anal. Ed., 12, 499 (1940).

25. J. H. Roe and E. W. Rice, J. Biol. Chem., 173, 507 (1948).

26. G. Ceriotti, J. Biol. Chem., 180, 59 (1955).

27. E. Wortheim Textbook of Organic Chemistry, 3rd ed., McGraw-Hill, New York, 1951.

28. C. R. Noller, Chemistry of Organic Compounds, W. B. Saunders Co., Philadelphia, 1957.

29. A. H. Brown, Arch. Biochem., 11, 269 (1946).

30. A. Meister, Biochemistry of Amino Acids, Academic Press, New York, 1957.

31. J. R. Whitaker and F. E. Deatherage, J. Am. Chem. Soc., 77, 3360 (1955).

32. S. Matsushita and F. Ibuki, Mem. Research Inst. Food Sci. Kyoto Univ., 22, 32 (1960).

33. M. L. Bazinet and C. Merritt, Jr., Anal. Chem., 34, 1148 (1962).

34. J. E. Lovelock, Anal. Chem., 33, 162 (1961).

35. R. J. Goodlow and F. A. Leonard, Bact. Rev., 25, 182 (1961).

36. N. H. Furman, Anal. Chem., 22, 1561 (1956).

37. A. Zlatkis and J. F. Oro, Anal. Chem., 30, 1156 (1958).

38. J. Janak, Gas Chromatography, A Symposium (ed. by R. P. W. Scott), p. 387, Butterworths, Washington, 1960.